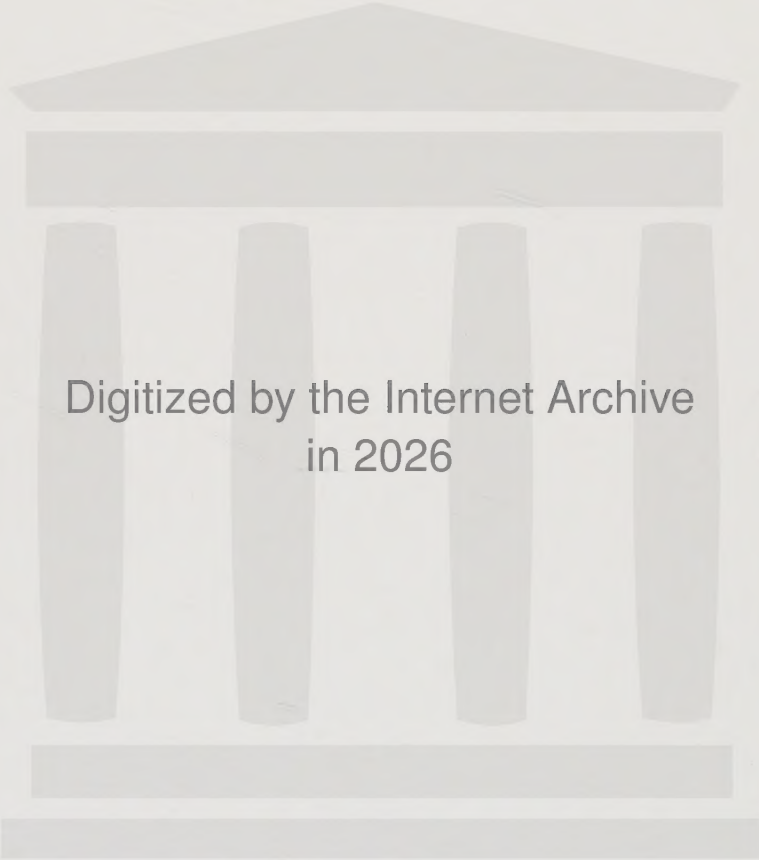


Pancreatic Colipase

**Structure-function relations and
the trypsin activation of its proform**

Tadeusz Wieloch

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PANCREATIC COLIPASE
STRUCTURE-FUNCTION RELATIONS AND
THE TRYPSIN ACTIVATION OF ITS PROFORM

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Abstract Pancreatic procolipase is secreted by the pancreas activated in the intestinal lumen and participating in the duodenal degradation of the dietary fat as a cofactor to pancreatic lipase. High resolution proton nmr studies of porcine procolipase and colipase and horse procolipase are presented. The tyrosine rich region is structurally described and defined as the micell binding site on colipase. The ionization state of the α -amino group is important for the binding of (pro)colipase to a tributyrine-bile salt interface. Trypsin activation of procolipase yields a molecule devoid of a N-terminal pentapeptide and with better lipid binding and lipase activating properties on charged and zwitterionic substrate interfaces. No gross conformational changes could be detected by ^1H - and ^{13}C -nmr during trypsin activation. A local change in the environment of the α -amino group and the exposure of the hydrophobic sequence -Gly ₆ -Ile ₇ -Ile ₈ -Ile ₉ - could explain the trypsin activation effect. A model for the colipase interaction with a triglyceride interface covered with charged amphiphiles is discussed. Product activation of lipolysis by pancreatic lipase was observed at conditions of high surface pressure on rac-1,2-dilaurin monomolecular films. Calcium ions do not bind to (pro)colipase as studied by ^{43}Ca -nmr. The physiological implications of the product activation of lipolysis the trypsin activation of procolipase and the importance of the basic and hydrophobic residues on colipase are discussed.			
Key words Colipase, pancreatic lipase, duodenal triglyceride hydrolysis, bile salt micelles, ^1H -nmr, ^{13}C -nmr, ^{43}Ca -nmr, monolayer technique, product activation, trypsin activation, interfacial binding			
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Date Lund, May 6, 1981

From the Department of Physiological Chemistry
University of Lund, Lund, Sweden

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To

Marianne, Mattias and Maria

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LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. An nmr study of a tyrosine and two histidine residues in the structure of porcine pancreatic colipase.
Tadeusz Wieloch and Karl-Erik Falk
FEBS Lett. 85 (1978) 271-274
- II. High resolution proton magnetic resonance study of porcine colipase and its interactions with taurodeoxycholate.
Tadeusz Wieloch, Bengt Borgström, Karl Erik Falk and Sture Forsén
Biochemistry 18 (1979) 1622-1628
- III. Is there a hydrophobic aromatic domain on porcine colipase?
Tadeusz Wieloch
Submitted for publication
- IV. Evidence for a pro-colipase and its activation by trypsin.
Bengt Borgström, Tadeusz Wieloch and Charlotte Erlanson-Albertsson
FEBS Lett. 108 (1979) 407-410
- V. Trypsin activation of porcine pancreatic procolipase. Determination of K_m and k_{cat} and the importance of the α -amino group.
Tadeusz Wieloch
Submitted for publication
- VI. Porcine pancreatic procolipase and its trypsin activated form. Lipid binding properties and lipase activation on monomolecular films.
Tadeusz Wieloch, Bengt Borgström, Gerard Pieroni, Franc Pattus and Robert Verger
FEBS Lett. In press.

VII. Product activation of pancreatic lipase. Lipolytic enzymes as probes for interfaces.

Tadeusz Wieloch, Bengt Borgström, Gerard Pieroni,
Franc Pattus and Robert Verger

Submitted for publication

VIII. Calcium binding to porcine pancreatic phospholipase A₂ studied by $^{43}\text{Ca}^+$ nmr.

Thomas Andersson, Torbjörn Drakenberg, Sture Forsén,
Tadeusz Wieloch and Mats Lindström

FEBS Lett. 123 (1981) 115-117

ABBREVIATIONS

nmr	- nuclear magnetic resonance
TDC	- taurodeoxycholate
BSA	- bovine serum albumin
di-C ₁₂ -PG	- didodecanoylphosphatidylglycerol
rac-1,2-dilaurin	- rac-1,2-didodecanoylglycerol
PLA ₂	- phospholipase A ₂
PLA ₁	- phospholipase A ₁

INTRODUCTION

The digestion and absorption of fat is a rapid and highly efficient process. After feeding human subjects with test meals, it was found that more than 95% of the fat was absorbed in the proximal jejunum (Borgström *et al.*, 1957). The efficiency of this process is achieved by optimizing different steps in digestion such as

- the emulsification of the substrate
- the enzyme secretion by the pancreas
- the admixing of bile
- the efficiency of the enzymatic processes
- the transport of products in the aqueous phase
- the absorption of the products by the intestinal mucosa

The composition of the small intestinal contents, from the physico-chemical point of view, is characterized by an oil phase composed of the dietary di- and triglycerides including nonpolar lipids such as fat soluble vitamins and cholesterol-esters and a water phase composed of amphiphilic lipids known to form aggregates such as micelles, liposomes or liquid crystals in water. The latter group includes the phospholipids, bile salts and products of lipolysis, *i.e.* fatty acids and monoglycerides. As a consequence of the amphiphilic character of these lipids, they will partition between the oil and micellar phases according to their tensioactive properties. This will result in an excessive accumulation of amphiphiles at the triglyceride/water interface.

The main triglyceride hydrolyzing enzyme in the small intestine of man and most higher animals is pancreatic lipase. Its ability to hydrolyze triglycerides, is restricted to an environment where the concentration of the tensioactive substances is low. In the situation present in the small intestinal lumen the lipase molecule will not reach its substrate because of the presence of bile salts and other amphiphiles, accumulated at the substrate interface. To overcome this interfacial barrier, a small protein, colipase, is secreted with the pancreatic juice into the duodenum. This molecule has been

shown to be an absolute requirement for pancreatic lipase to hydrolyze triglycerides under the conditions in the small intestinal lumen.

The present investigation throws some light on the function of colipase on the molecular level, with special reference to its lipid binding properties and the importance of the trypsin activation of its proform. It also deals with the modulation of the substrate interface as a mechanism for making the triglyceride available for lipase hydrolysis.

BACKGROUND

Gastric lipolysis

Fat digestion is a process already started in the pharynx by the action of the lingual lipase. This enzyme, secreted by the lingual serous glands, is able to hydrolyze up to 30% of the dietary triglycerides to mainly diglycerides (for a recent review on this subject see Hamosh, 1979). This initial hydrolysis has two important consequences for fat digestion. It enhances emulsification of the dietary fat. The products of lipolysis, *i.e.* the fatty acids and monoglycerides together with bile salts are good emulsifiers at low shear rates (Linthorst *et al.*, 1977). This emulsification increases the total substrate surface available for pancreatic lipase and consequently a faster degradation of the diglycerides and remaining triglycerides. The importance of this enhanced emulsification by the lingual lipase was demonstrated by Roy *et al.* (1979) who found that rats with esophageal fistula had a substantially decreased fat absorption compared with the normal animal, which could be restored if the fat was given in an emulsified form.

Secondly, the products of the gastric lipolysis of the triglycerides transforms the glyceride interface, enhancing the lipolysis by pancreatic lipase (Olivecrona *et al.*, 1973). This enhancement was demonstrated by Plucinski *et al.* (1979) who

found that the *in vitro* lipolysis of milk by pancreatic lipase increased if the milk was preincubated with lingual lipase.

Recently, Borgström (1980) using a commercial phospholipid stabilized triolein emulsion, Intralipid, as substrate for pancreatic lipase, observed a long lag time preceding the hydrolysis at a high rate. This lag time could be decreased by the addition of fatty acids into the incubation medium as well as increasing the enzyme concentration.

Duodenal lipolysis

The main enzyme responsible for intestinal lipolysis of dietary glycerides is pancreatic lipase (E.C. 3.1.1.3). This enzyme specifically hydrolyzes the 1 and 3 acyl bonds of triglycerides to fatty acids and 2-monoglycerides (Borgström, 1954; Mattson and Beck, 1956). As lipase cannot exert its catalytical role under the conditions prevailing in the duodenal lumen without the presence of colipase, much attention has been focused on the latter molecule during the past 10 years. Many comprehensive reviews have recently been written on this subject (Brockerhoff, 1974; Borgström *et al.*, 1979; Sémériva and Desnuelle, 1980). The following presentation briefly summarizes the development in the colipase field.

The first indication of the existence of a colipase molecule was reported in 1910 by Rosenheim who found a heatstable cofactor to the lipase activity of the pancreas. Since this first colipase observation and its rediscovery by Baskys (1963) a colipase has been purified from pig (Maylié *et al.*, 1973; Erlanson *et al.*, 1973; Canioni *et al.*, 1977), cow (Rathelot *et al.*, 1975), man (Sternby and Borgström, 1979), horse (Julien *et al.*, 1978; Chapus *et al.*, 1980) and chicken (Bosc-Bierne *et al.*, 1981).

Colipase has a highly conserved primary structure (Fig. 1). This is probably why different colipase molecules are able to activate lipases from different species (Sternby and Borgström, 1981). Most of the colipase research has been conducted on porcine colipase.

		10		20		30																																
Pig	V	P	D	P	R	G	I	I	I	N	L	D	E	G	E	L	C	L	N	S	A	Q	C	K	S	N	C	C	Q	H								
Horse	:	:	:	:	:	V	:	:	:	:	E	A	:	:	I	:	M	:	:	:	:	:	:	:	:	:	E	:	:	H	R							
Chicken	:	:	:	:	:	L	:	:	F	:	:	:	T	:	:	:	✕	:	Q	:	:	:	:	:	:	:	E	:	:	:	E							
Man	:	:	:	:	:	I	:	:	:	E	N	:	:	:	:	✕	M	:	:	:	:	:	:	:	:	:	:	:	:	:	:							
						40										50														60								
Pig	D	T	I	L	S	L		R	C	A	L	K	A	R	E	N	S	E	C		A	F	T	L	Y	G	V	Y	Y	K	C	P						
Horse	E	S	S	:	:	:	A	:	:	:	A	:	:	:	:	:	S	:	:	:	:	S	:	W	:	:	:	:	:	:	:	:						
Chicken	:	S	G	:	:	:	A	✕	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:						
						70										80														90								
Pig	C	E	R	G	L	T	C	E	G	D	K	S	L	V	G	S	I	T	N	T	N	F	G	I	C	H	N	V	G	R	S	A	S					
Horse	:	:	:	:	:	:	:	:	:	V	:	:	:	:	:	:	T	:	:	:	:	:	:	:	:	:	:	:	:	:	F	D	A	A	:	:	E	✕

Figure 1. The amino acid sequence of colipase from pig (Erlanson *et al.* 1973, Charles *et al.* 1974), horse (Bonice *et al.* 1981), chicken (Bosc-Bierne 1981) and man (Sternby *et al.* 1979). The residues marked (✕) reflect homologies with the sequence of pig colipase.

This molecule was first purified in a partially degraded form with a glycine (Gly₆, Figure 2) N-terminal. The colipase molecule purified under protective conditions, however, has an additional pentapeptide Val₁-Pro₂-Asp₃-Pro₄-Arg₅- in the N-terminal end. This colipase will be referred to as procolipase in the text. The primary structure of a partially degraded colipase molecule and the position of three out of five disulfide bridges has been determined (Charles *et al.*, 1974; Erlanson *et al.*, 1974) (Fig. 2). The five disulfide bonds give the molecule an appearance of a tight structure with a central core and two tails in the N-terminal and C-terminal part. The three-dimensional structure of colipase has not yet been elucidated. The structural similarities of colipase with the phospholipase (Haas *et al.*, 1970) and the snake toxins (Lauterwein *et al.*, 1978) are striking. The high content of disulfide bridges, the molecular weights, and the interactions with interfaces are some common features further discussed in the next sections.

pancreatic juice before entering into the duodenum. In the bile a complex of lecithin, bile salts and a protein is suggested to form a structure called the bile lipoprotein complex. Colipase is first bound to this complex and then lipase binds to colipase. This entity will in turn adsorb to the triglyceride surface (Lairon *et al.*, 1978). Although the sequences of events differ in the models, in both models the binding of colipase to an interface is an obligatory step for lipase to reach its substrate.

THE PRESENT INVESTIGATION

Some structural features of colipase

A variety of methods can be applied to obtain information about the structure/function relations of proteins. Specific chemical modification of amino acid side chains and limited proteolysis can give information about the importance of certain amino acids or larger sequences in the protein for its structure or properties. Yet another strategy is to use spectroscopic methods, by taking advantage of some intrinsic property of the macromolecule for these kind of studies. In the present investigation proton and carbon-13 magnetic resonance spectroscopy has been applied in order to gain some structural information of the colipase molecule in solution as well as to study its interactions with ligands.

Nuclear magnetic resonance spectroscopy (nmr). NMR is a spectroscopic method (Wütrich, 1976) based on the nuclear magnetism of certain atomic nuclei, such as protons and carbon-13. This section gives a simplified explanation of some nmr parameters used in the text. In a magnetic field an excess of the nuclei are in a low energy state and may be brought to a high energy state by absorption of a specific irradiated radio frequency. This absorption is detected and visualized as a resonance in the nmr spectrum. The following parameters are obtained from the nmr spectrum. The chemical shift value in ppm is the position of the resonances on the frequency

scale. This position is dependent on the magnetic environment of the nuclei. The resonance can be split into multiplets by the spin-spin coupling with other nuclei mediated through the covalent bonds in the molecule. Using more sophisticated pulse nmr-techniques, relaxation rate constants can be obtained. T_1 - the longitudinal relaxation time - is related to the dynamics of the protein structure. T_2 - the transversal relaxation time - can be obtained from the linewidth of the nmr resonance at half height and reflects the rotational and translational motion of the molecule. The linewidth of a resonance can also be affected by chemical exchange processes. If a nucleus can experience two magnetic environments, it will have two resonance frequencies one for each environment. If the life-time in one environment is long enough, two nmr resonances will be observed (slow exchange limit). If the nuclei is changing environment fast, an average chemical shift value will be obtained (fast exchange limit). Intermediate exchange rates increase the linewidth of the resonance(s).

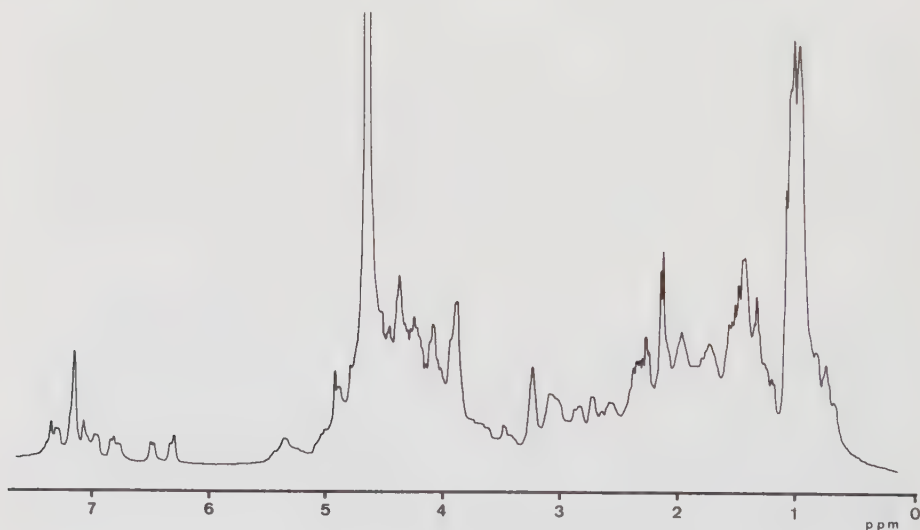


Fig. 3. The proton nmr spectrum of horse colipase B at pH 11.2.

Ions such as ^{43}Ca , ^{25}Mg , and ^{23}Na have also nmr sensitive nuclei. Although other relaxation mechanisms dominate these nuclei, similar information can be obtained as for protons and carbon-13.

Proton nmr studies of procolipase. Figure 3 shows a proton nmr spectrum of horse colipase. The high field portion of the spectrum (0-5 ppm) contains proton resonances of the $-\text{CH}_3$, $-\text{CH}_2$, and $-\text{CH}$ groups. In the low field region (5-10 ppm), the proton resonances of the aromatic protons of the tyrosine phenylalanine, tryptophan residues and the C_2 and C_4 protons of the histidine residues are observed.

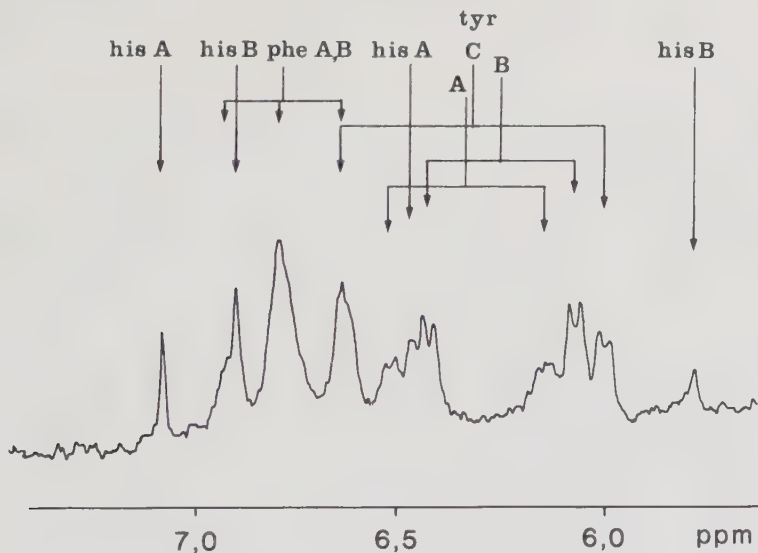


Fig. 4. The aromatic region of the proton nmr spectrum of pig colipase at pH 12.1.

The high field portion of the procolipase spectrum is more complicated than the low field portion, and individual resonances apart from the methionine methyl groups are difficult to assign in the absence of a three dimensional structure. The low field region has fewer resonances. Figure 4 shows this region of pig procolipase. The three tyrosines and one phenylalanine residue in the octapeptide sequence

Phe₅₀-Tyr₅₇ exhibit their resonances in this region and thus allow a closer study of this part of the colipase molecule. The two histidines at position 30 and 86, may serve as probes for other parts of the molecule.

The apparent pK'_a values of the histidines in pig procolipase and colipase were determined to 6.9 and 7.8. A small inflection of the titration curve at pH 5.2 and the high pK'_a value of His-A (I) would indicate that a negative charged residue with a pK'_a value of ≈ 5 could be positioned in the vicinity of this histidine. A tentative assignment was thus made to His-30 in the structure, as Asp-29 could correspond to the negative charge affecting the pK'_a value of this histidine. The observed chemical shift effect of the Tyr-C ortho-resonance, when His-B ionizes, could be interpreted as a structural change accompanying the ionization and/or a ring current induced shift of the histidine imidazole ring. The latter explanation is favoured. The chemical shift effects were only observed on the Tyr-C ortho proton resonance suggesting an extremely local change of the magnetic environment. This was stressed in paper II where a similar shift effect on the histidin-B C₄-proton resonance was observed when Tyr-C was ionized. A structural change would probably change the shift of both the C₂ His-B and the Tyr-C meta protons and other tyrosine resonances too.

The structural information obtained from the proton nmr investigations of the histidine and tyrosine residues of pig procolipase can be summarized: Two tyrosines are surface tyrosines exposed to the solvent water with freely rotating aromatic rings (II). The third tyrosine is a "buried" residue with a hindred rotation of the aromatic ring (II). These findings are supported by fluorescence quenching studies showing that one tyrosine is not accessible to molecules larger than 2.8 Å, whereas at least one is accessible to larger molecules, such as glucose (Sari *et al.*, 1978). Furthermore, chemical modifications of the tyrosine residues using N-acetylimidazole resulted in the modification of two out of the three tyrosine residues (Erlanson-Albertsson, 1980).

Histidine B is closely related in space to one of the tyrosines with the planes of the rings parallel or close to parallel (I). It is partially buried, as the proton exchange rate is slow (I). His-A is a surface histidine. As a consequence of the tentative assignment of His-A to His-30 in the structure, His-B was assigned to His-86. This made us propose the formation of a hydrophobic-aromatic region on pig colipase composed of the sequences Phe₅₀-Tyr₅₇ and Phe₈₂-Val₈₈ (II).

Independently of the present work P. Cozzzone and collaborators conducted similar experiments. A similar proposal of a hydrophobic aromatic domain on colipase was reported (Key ref. Cozzzone *et al.*, 1981). Recently, however, two contradictory reports on the sequence of horse colipase have been published (Julien *et al.*, 1980; Granon *et al.*, 1981). This colipase molecule has only one histidine residue. Granon *et al.* (1981) further reported that this histidine was at position 29 whereas Julien *et al.* (1980) found an arginine at position 29. The latter sequence was only conducted up to residue 55. Paper III reports an nmr investigation of horse colipase B purified as reported by Bonicel *et al.* (1981) who confirmed the results of Granon *et al.* (1981) and further found a phenylalanine at position 86. The C₄ resonance of this sole histidine of horse colipase B clearly has similar properties as that of His-B in pig colipase spectrum (III). This would imply that His-B in pig colipase has to be reassigned to histidine-30 in the structure. The data obtained from the studies on horse procolipase thus could not verify the proximity of the sequences Phe₅₀-Tyr₅₇ and Phe₈₂-Val₈₈. A definite settlement of this issue must await the three-dimensional structure of colipase.

Interactions of colipase with interfaces

The micelle binding site on colipase. The interaction of bile salts with colipase is a well studied phenomenon. Gel filtration and equilibrium dialysis (Borgström and Donnér, 1975), UV-spectroscopy (Sari *et al.*, 1975) and ultracentrifugation studies (Charles *et al.*, 1975) have revealed that approximately one micelle of TDC binds to one colipase molecule. UV-

(Sari *et al.*, 1975) and CD-investigations (Donnér *et al.*, 1976) showed that the aromatic residues were affected by the TDC binding. The binding between the micelle and colipase is hydrophobic (Donnér *et al.*, 1976). The present ^1H -nmr data (II,III) shows that the surface tyrosine residues are interacting with the micelles of TDC and that a histidine is also affected, suggesting that a small conformational change takes place upon micelle binding. The tyrosine rich region is thus definitely a part of the bile salt binding site on colipase. TDC-oleic acid mixed bile salt micelles also interact with the tyrosine region of colipase in a similar manner to the pure TDC micelles (III). This might not seem surprising but there is a very important distinction between these two types of micelles on the effect on the binding between lipase and colipase. In the absence of pure TDC micelles the K_d between lipase and colipase is 10^{-6} M and increases in the presence of TDC micelles to 10^{-5} M (Donnér *et al.*, 1976). In the presence of TDC-oleic acid micelles, however, K_d is decreased to 10^{-8} - 10^{-9} M (Patton *et al.*, 1978).

The binding site on colipase for the two types of micelles is apparently similar (although it cannot be excluded that other parts on the molecule apart from the tyrosine rich region participate in the binding). The small structural change observed in the nmr spectrum when the TDC-micelles and TDC-oleic acid mixed micelles bind cannot be of critical importance for the binding of lipase to colipase as such large differences in the binding constants are observed. This difference probably lies in the binding of fatty acids to the lipase molecule.

The lipase molecule has been shown to reversibly catalyze the reactions of triglyceride hydrolysis as well as its synthesis (Borgström, 1964). Moreover, at $\text{pH} < 5$, it is possible to obtain a stable acylated active site of lipase (Chapus *et al.*, 1976). The lipase active site can thus readily be acylated by a fatty acid in the TDC-oleic acid mixed micelle. The difference in binding constants between lipase and colipase in the presence of TDC-micelles and TDC-oleic acid mixed micelles,

respectively, can now be rationalized. Colipase binds to the TDC-oleic acid mixed micelle. This complex will have two binding sites for lipase, one on colipase the other on the micelle. When lipase binds to colipase the active site is acylated by the fatty acid which act as a hydrophobic tail sticking into the micelle and stabilizing the complex (Figure 5).

Is the colipase-micelle interactions relevant for the events taking place at the substrate interface? The importance of polar interactions in the procolipase binding to the bile salt covered triglyceride interface were demonstrated by Borgström and Donnér (1977). They showed that 80% of the procolipase is desorbed from the interface at 0.5 M NaCl. Moreover at pH 9.0 and 0.15 M NaCl procolipase did not bind to this interface (Borgström, 1975). Chemical modification of the amino groups of procolipase with citraconic anhydride prevented colipase from binding to the interface (Erlanson *et al.*, 1977) indicating that the amino groups on procolipase are important for the binding of procolipase to the interface. Furthermore, if cationic bile salts are present at a tributyrine interface procolipase cannot bind (Borgström, 1977).

The amino groups of colipase can be methylated by reductive methylation using ^{13}C CHO and sodium boronhydride and studied by ^{13}C -nmr (V). By a pH/ ^{13}C -nmr study it is possible to determine the pK'_{a} values of the lysines and the N-terminal α -amino residue of procolipase (V). The pK'_{a} value the α -amino group of procolipase was found to be 7.4 while the lysyl residues were in the range 10-11. Considering the pH binding profile of procolipase to the TDC covered tributyrine interface (V) a good candidate for an essential positive charge would be the α -N-terminal amino group having a pH titration curve similar to the pH-binding curve of procolipase to the triburyin-TDC interface.

Procolipase is an acidic protein with a pI of 5.2. In order to interact with a negatively charged interface at pH 7.0 colipase seems to have an uneven charge distribution with a positive charge dominating the part of the molecule interacting with

the interfaces. The α -amino terminal could be one out of several important positive charges. If this residue is deprotonized the dipolar character of procolipase might be abolished and make the molecule unable to bind to the TDC covered interface.

Although the electrostatic interactions are important other forces evidently also contribute to the binding. For example, a TDC-trioctanoyl ether interface is not recognized by procolipase (Borgström and Donné, 1977). Based on this, it has been suggested that hydrogen bonds between procolipase and the carboxyl group(s) of the ester linkage might be formed. In addition, the surface density of bile salts may be lower allowing both hydrogen bonding as well as a hydrophobic effect at the interface to take place. The tyrosines of the hydrophobic region of the micelle-binding site on colipase (II,III) could account for such effects. In fact, the acetylation of the two surface tyrosines of colipase abolished its ability to activate lipase on an Intralipid based assay (Erlanson-Albertsson, 1980). But these interaction must be superficial, as the tensioactivity of colipase is low and no large increase in surface pressure could be detected on phospholipid monomolecular films when colipase was adsorbed (Verger *et al.*, 1977; VII).

Trypsin activation of porcine procolipase

Evidence for the existence of a procolipase. Limited proteolysis is a common mechanism observed in the regulation of biochemical events such as for example, in blood coagulation (Davie *et al.*, 1979), the maintenance of blood volume and pressure by the renin- and angiotensin system as well as the complement cascade (Müller-Eberhardt, 1975). The far the most studied system is the activation of the pancreatic zymogens (Kassel *et al.*, 1973). The intestinal digestive enzymes responsible for the protein and phospholipid digestion are stored in the pancreatic acinar cells as inactive zymogens and secreted in the zymogen form into the pancreatic duct (Greene *et al.*, 1963). In the brush border trypsinogen is

first cleaved by enterokinase to trypsin. The trypsin then activates the other zymogens; chymotrypsinogen, procarboxypeptidase, prophospholipase and proelastase. The activation includes the cleavage of one or several bonds in the proteins, usually in the N-terminal part. The reason for keeping the enzymes in a proform is their potential degradative properties in the active forms, which could be lethal for the pancreatic cells as well as the surrounding tissues. The zymogens are stored in subcellular structures called zymogen granulae. These granulae also contain colipase and lipase activities (Arnesjö *et al.*, 1968).

The procolipase purified from the pancreatic gland is thus likely to be stored in these granulae. This colipase molecule has the following N-terminal sequence: Val₁-Pro₂-Asp₃-Pro₄-Arg₅-Gly₆- (Erlanson *et al.*, 1973). Procolipases containing 107 (Erlanson *et al.*, 1973), 101 (IV) and 93 amino acids (V) have been reported. All differ in the C-terminal end but have a N-terminal valine. The sensitivity of the Arg₅-Gly₆ bond for trypsin accounts for the shorter forms obtained in earlier purifications (Charles *et al.*, 1974). Colipase A and B purified from horse (Julien *et al.*, 1980; Chapus *et al.*, 1980) both have this N-terminal sequence. The N-terminal residue of the chicken and human colipase on the other hand, were found to be a glycine (Figure 1). The fact that the Arg₅-Gly₆ bond is an extremely good substrate for trypsin (IV), could account for proteolysis during the purification although measures were taken during the purification of chicken colipase to inhibit proteolytic activity (Bosc-Bierne *et al.*, 1981).

In the pancreatic juice, the proteolytic enzymes were found to be in their zymogen forms (Greene *et al.*, 1963). Although this being an indirect evidence it is reasonable to assume that the colipase molecule is secreted into the pancreatic duct with an intact N-terminal sequence.

The zymogens are activated in the small intestinal lumen by the action of trypsin. The K_m and k_{cat} values for these reactions has been determined for trypsinogen and chymotrypsinogen (Abita

et al., 1969) and for phospholipase A₂ (Abita *et al.*, 1972; Nieuwenhuizen *et al.*, 1973). The K_m and k_{cat} values of the trypsin cleavage of the Arg₅-Gly₆ bond of procolipase are in the same order of magnitude as that observed for these zymogens (V). Moreover, native bile, bile salts micelles, bile salt-lecithin and bile salt-fatty acid mixed micelles do not interfere with this activation (V), although these structures bind colipase with K_d = 10⁻⁴-10⁻⁹ M (Lairon *et al.*, 1978; Patton *et al.*, 1978).

The physiological active form of colipase is thus likely to be a form devoid of the N-terminal pentapeptide.

What are the effects of the trypsin cleavage of the N-terminal pentapeptide on lipolysis? The activating effect of the limited proteolysis of procolipase by trypsin was first observed in an assay based on a phospholipid covered triolein emulsion, Intralipid (IV), as a decrease in a lag time τ_d (VII) preceding a high hydrolytic rate. Under the conditions prevailing in the small intestinal lumen, the τ_d for procolipase was 15 min, whereas that of colipase was 0.8 min. In a system composed of a monomolecular films of rac-1,2-dilaurin in the presence of 2 mM TDC at 40 dynes/cm colipase could activate lipase while procolipase could not (VI). Apparently colipase is able to activate lipase on substrate interfaces at high surface pressure. In a tributyrine-TDC based assay at pH 7.0, the colipase activity was 1.2-1.5 times higher for colipase than procolipase (IV). At higher pH the difference in activity increases. At pH 8.8 the specific activity of procolipase was 3000 U/mg whereas that of colipase was 18,000 U/mg (V).

What are the molecular effects of the trypsin activation?

First, recall that colipase has two binding sites: One for the substrate interface and one for pancreatic lipase. The trypsin activation could thus affect either or both of these binding sites.

Differences are observed in the binding of procolipase and colipase to monomolecular films and triglyceride-TDC disper-

sions. This difference in binding is in the order of a factor of two on the negatively charged di-C₁₂-PG and zwitterionic egg-PC films (VII). On the neutral di-C₁₂-G films no difference is observed (VII). The pH dependence of the binding to tributyrine-TDC dispersions shows a shift of an apparent "pK" value of the binding to the interface from 7.3 for procolipase to 8.1 for colipase (V). Apparently the ionization of a basic residue(s) is altered by the activation of procolipase. No gross structural effects were observed by ¹H-nmr and ¹³C-nmr (V). The pK_a values of the histidines, tyrosines and lysines are not changed upon activation (V). The N-terminal α-amino group changes magnetic environment and pK_a value upon activation (V). This implies that the change of the ionization state of the α-amino group contributes to the better binding of colipase to the charged lipase substrate by a better position in relation to the negative potential of the interface (V).

No differences were observed in the penetration properties into the hydrocarbon part of the monolayer of colipase compared with procolipase (VII). This does not of course, exclude a hydrophobic effect, taking place superficially at the interface.

The binding of lipase to colipase in the absence of an interface was not affected by the activation (IV). Using rac-1,2-dilaurin-TDC mixed films at 37.5 and 40 dynes/cm lipase was fully activated with colipase, whereas the activity was negligible or absent in the presence of procolipase (VI). An inverse relation of the decreased binding of colipase and increased lipase activity with increased surface pressure raises the question whether the binding between lipase and colipase at the interface also is affected by the trypsin activation (VI).

What are the physiological implications of the trypsin activation of procolipase? The trypsin activation of procolipase is another way of making fat digestion more efficient. Under physiological conditions, lipase and colipase are never confronted with a pure triglyceride interface. Bile salts, lipolytic products, and phospholipids are adsorbed to the dietary fat form-

ing highly packed charged interfaces. Any mechanism, for example, trypsin activation of procolipase, increasing the binding of colipase to such interfaces is to the purpose of fat digestion.

Why must then colipase be stored as a proform if its tenstio-activity does not change with trypsin activation? The latteral pressure of erythrocyte membranes has been found to be 30-34.7 dynes/cm (Demel *et al.*, 1975). If we presume that the surface pressure of the zymogen granulae membranes is in that order of magnitude, the activated colipase would be able to bind to the membrane (VII). As lipase is also found in the zymogen granulae (Arnesjö *et al.*, 1968) it could bind to colipase and thus reach the ester bonds of the phospholipids. Pancreatic lipase has an inherent PLA_1 -activity (Haas *et al.*, 1965), which could be dangerous for the zymogen granulae membrane.

Product activation of lipolysis

The concept of product activation of lipolysis might seem a tritle challenging if one recalls the well known and studied product inhibition of lipolysis (Brockerhoff, 1974). The difference between these two phenomena must be looked for at the different interfaces presented to the enzyme and the presence of product acceptors and cofactors.

The inhibition of lipase hydrolysis of long chain triglycerides in the absence of bile salts and Ca^{2+} has been proposed to take place by several mechanism.

The lipase catalyzed hydrolysis of triglycerides is a reversible reaction (Borgström, 1964). As products accumulate the reaction is shifted towards synthesis. An excessive accumulation of products will further increase the surface pressure of the interface thus depleting the interface from triglycerides (Scow *et al.*, 1979) and also causing desorption of lipase.

Addition of bile salts to this system will partition the fatty acids between the oil phase and the micellar phase (Hofmann and Borgström, 1962) driving the lipase reaction towards hydro-

lysis (Borgström, 1964). Addition of Ca^{2+} will precipitate the fatty acids as calcium soaps and thus increase the rate of hydrolysis (Benzonana and Desnuelle, 1968). In the absence of product acceptors the substrate interface is thus initially characterized as a surface of high surface tension. As products are formed the surface tension decreases (surface pressure increases) causing desorption of lipase and depletion of substrate. In the case of product activation of lipolysis the initial conditions are reversed.

Product activation of triglyceride lipolysis by pancreatic lipase was first observed in an assay composed of a phospholipid stabilized triolein emulsion, Intralipid (Borgström, 1980). This hydrolysis was characterized by biphasic kinetics. Initially a low hydrolytic rate was observed followed by a sudden increase in hydrolysis after a certain lag time. Similar biphasic kinetics were observed in the monolayer system using rac-1,2-dilaurin as substrate (VII). The time elapsed between enzyme injection and the sudden increase in hydrolytic rate was named τ_d (lag time product dependent) which can be used as a measure of product activation. The biphasic kinetics were only observed at high surface pressure, 40 dynes/cm, whereas at low surface pressure, 28 dynes/cm, simple, "classical" kinetics are characterized by τ_i (enzyme concentration independent lag time) (VII).

High lipid packing (surface pressure) is thus a prerequisite for observing these biphasic kinetics *i.e.* product activation. At high surface pressure and in the absence of colipase, lipase is partitioned in favour of the bulk (Borgström, 1980). On the Intralipid emulsion droplet only a few percent of the interface is covered by triglycerides (Pieroni and Verger, 1979). The interface is thus largely depleted of substrate. The activation of lipolysis is interpreted as an increased binding of colipase to the interface and a subsequent increase in lipase binding (VII) due to a time dependent change of the interface as products are formed (VII). This should cause inhibition, but the products only stay transitorily at the interface. The high surface pressure increases the rate of

fatty acid desorption (Scow *et al.*, 1979). Moreover, the presence of Ca^{2+} ions is a prerequisite for hydrolysis to take place (Borgström, 1980; VII) and the fatty acids present at the interface probably form Ca^{2+} soaps preventing the reacylation of glycerides (Borgström, 1954).

Addition of BSA desorbs the products from the interface increasing τ_d . At 40 dynes/cm, BSA by itself is not adsorbed to the film (Stållberg and Torell, 1939). Factors increasing the concentration of products at the interface will correspondingly decrease τ_d . For example by adding oleic acid and monoolein, increasing the colipase and lipase concentration. The most profound decreasing effect on τ_d is observed when calcium ions are added.

Recently it was reported that microheterogeneities of fatty acids in monomolecular films of phosphatidylcholine might exist (Hauser *et al.*, 1979). Furthermore, calcium-fatty acid soaps in stearic acid monolayer films were observed to form small clusters (Neuman, 1975).

The effectiveness of calcium ions in product activation could be pictured as the formation of such calcium-fatty acid clusters at the interface (VII). At the boundaries of these clusters local defects in lipid packing and an increase in the negative potential of the interface causes an increased binding of colipase.

Another alternative could be a binding of calcium ions between colipase and the fatty acid. We have adopted the ^{43}Ca -nmr method of measuring the calcium binding to procolipase and colipase. It was possible to obtain Ca^{2+} binding constants, calcium exchange rate, and the pK_a values for the Ca^{2+} binding groups of prophospholipase A_2 by this method (VIII). In 2 mM solution of procolipase and colipase, respectively, and 2 mM Ca^{2+} , no increase in the linewidth was observed indicative of a low binding constant. The calcium effect most certainly is an effect on the interfacial constituents.

Biphasic kinetics and product activation have been observed during the action of PLA_2 from *Crotalus Atrox* (Tinker *et al.*, 1978), *Crotalus durissus Terrificus* (Roholt *et al.*, 1961) and of phospholipase D (Quarles *et al.*, 1969) on their substrates at high surface pressure.

Apart from being an interesting enzymatical and kinetical problem, the product activation of lipolysis might be of great importance for the initiation of duodenal fat digestion as already commented in the Introduction. Some of the dietary fat such as milk or cream is emulsified and the particles are covered by one or several layers of phospholipids. The lingual lipase by some mechanism can reach and partially hydrolyze the triglycerides. This interface is thus "activated" by the products of gastric lipolysis when it is presented as substrate for pancreatic colipase/lipase.

The implications of the present results on the mechanism of the intestinal lipolysis by pancreatic lipase

Figure 5, bottom, shows a hypothetical picture of the interactions of procolipase and colipase with the triglyceride interface covered with negatively charged or zwitterionic amphiphiles. The negative charges attract the basic residues on colipase orienting the tyrosine rich region and the N-terminal end towards the substrate interface. At the interface the interaction is stabilized by hydrogen bonds and/or a hydrophobic effect between these regions on colipase and the hydrocarbon parts of the interface. The trypsin activation reveal a new α -amino group (V) in a better position to interact with the negative charges at the interface. The hydrophobic sequence Gly₆-Ile₇-Ile₈-Ile₉- (Figure 2) will further stabilize the interaction by a superficial hydrophobic effect (VII). The better binding of colipase compared to procolipase on monolayers at high surface pressure can be explained by these effects (VII). As the binding between lipase and colipase is mainly hydrophobic (Donnér *et al.*, 1976) an local change in this hydrophobic sequence could also affect the lipase-colipase binding at the interface (VI).

A similar picture of the binding of cardiotoxin II from *naja mossambica mossambica* to negatively charged phospholipid interfaces has been hypothesized (Lauterwein and Wütrich, 1978). Although, in this case a true penetration of the protein into the hydrocarbon interior of the interface takes place (Bougis *et al.*, 1981) the interaction is sensitive to high salt concentration and high pH suggesting that the basic amino acids of the cardiotoxin interact with the negatively charged phospholipids (Dufourcq and Foucon, 1978).

Another similarity with colipase is observed in the binding of pancreatic phospholipase A₂ to phospholipid interfaces. The α -amino group is important for the enzyme activity (Abita *et al.*, 1972). A negatively charged phospholipid is a better substrate for phospholipase A₂ than the zwitterionic (Pattus *et al.*, 1979). Furthermore, the crystal structure of pancreatic phospholipase A₂ shows that an excess of hydrophobic and basic amino acid residues are surrounding the active site of the enzyme (Dijkstra *et al.*, 1981).

In the intestinal lumen colipase will thus partition between the substrate interface and the micellar phase according to its affinity for the different interfaces. In the presence of pancreatic lipase, however, a high affinity binding complex (Patton *et al.*, 1978), interface-colipase-lipase-interface, is formed whenever triglycerides or lipolytic products *i.e.* fatty acids (and monoglycerides?) are present at the interface. Their presence makes an interaction between lipase and the interface possible, by an acylation of the active site or a binding to the essential serine outside the active site (Sémériva and Desnuelle, 1980), in contrast to other interfaces such as pure bile salt micelles. This will partition colipase in favour of the lipase substrate interface. The possible presence of small amounts of fatty acids in the bile lipoprotein complex (Lairon *et al.*, 1978) could account for the high affinity binding of colipase and lipase to this complex.

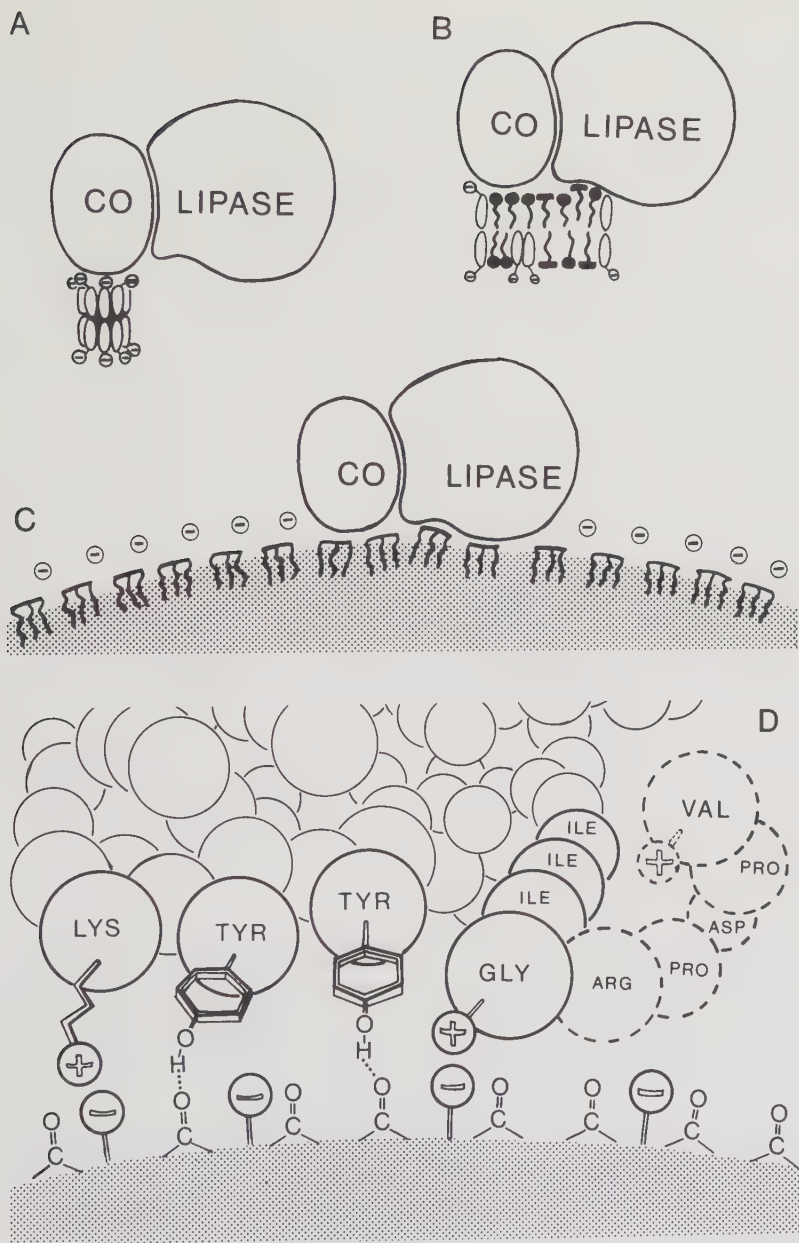


Figure 5. A hypothetical picture of the colipase/lipase interactions with:
 A. a bile salt micelle, B. a bile salt-fatty acid-monoglyceride mixed micelle, C. the triglyceride interface covered with negatively charged amphifiles (bile salts, phospholipids, fatty acids). D. The (pro)colipase binding to a triglyceride interface covered with negatively charged lipids.

SUMMARY

1. The structural and the dynamic properties of the tyrosine and histidine residues of pancreatic colipase have been investigated by proton nmr, and the existence of a hydrophobic aromatic region on colipase is discussed. The tyrosine rich region Tyr₅₃-Tyr₅₇ binds bile salt micelles and bile salt-fatty acid mixed micelles. The effects of the micelle-colipase interaction on the binding of lipase to colipase is discussed.
2. Carbon-13 nmr investigation of enriched methylated procolipase and colipase has been performed and apparent pK'_a values of individual amino groups are determined. From binding studies, it was concluded that the ionization of the N-terminal amino group is important for the binding of procolipase and colipase to a bile salt covered interface.
3. A procolipase containing 93 amino acid residues and with a N-terminal valine was purified from pig pancreas. This colipase is activated by trypsin by a cleavage of a N-terminal pentapeptide, Val₁-Pro₂-Asp₃-Pro₄-Arg₅- with a K_m of 0.06 mM^{-1} and k_{cat} of 3 sec^{-1} . The trypsin activation was not affected by bile salt micelles or bile. The activated colipase has better lipase activating and lipid binding properties on negatively charged and zwitterionic interfaces at high surface pressure.

The activation does not induce any gross conformational change as studied by nmr and UV-spectroscopy. A local effect in the N-terminal region by the exposure of the hydrophobic residues Gly₆-Ile₇-Ile₈-Ile₉- and a subsequent change in the ionization constant of the N-terminal amino group are suggested as increasing the affinity of colipase for the charged interfaces. A hypothetical model for the colipase interaction with a triglyceride interface covered with charged amphiphiles is presented and the physiological relevance of the trypsin activation discussed.

4. Product activation of pancreatic lipase in the presence of colipase was observed during the hydrolysis of a rac-1,2-dilaurin monomolecular film at a surface pressure of 40 dynes/cm. This activation was interpreted as a transitory stay of products at the interface, fatty acids, and monoglycerides, increasing the binding of colipase due to a change in the charge, and the formation of micro-heterogeneities at the interface.
5. No binding of calcium ions to procolipase and colipase could be detected by ^{43}Ca -nmr.

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AN NMR STUDY OF A TYROSINE AND TWO HISTIDINE RESIDUES IN THE STRUCTURE OF PORCINE PANCREATIC COLIPASE

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1. Introduction

Pancreatic colipase is a small protein of approx. mol. wt 11 000 [1]. Its primary structure and the position of three of the five disulfide bridges have been determined, while there is still ambiguity about the position of the additional two [2,3]. The crystal structure of colipase has not been reported.

Colipase participates in the hydrolysis of triglycerides in the duodenal lumen as a cofactor to the active enzyme, pancreatic lipase [4]. Its primary function is to counteract the desorbing effect of bile salts on lipase by anchoring the lipase molecule to the triglyceride surface [5,6]. The interactions between colipase and the triglyceride surface in the presence of bile salts has been shown to be of polar nature [7]. It is thus of interest to investigate the ionizable side-chains in colipase which might be responsible for the colipase-triglyceride interaction.

NMR can be a powerful tool in the studies of the conformation of proteins in solution [8] and in the determination of the pK_a values of specific amino acid residues in proteins, such as histidines, tyrosines and lysines [9]. This paper is the first in a series of high resolution NMR investigations on the structure and properties of colipase. In this investigation we have determined the pK_a values of the two histidines in colipase and made a tentative assignment of the

resonances to the amino acids in the sequence. We also conclude that one of the tyrosines and one histidine residue are closely related in space.

2. Experimental

Porcine pancreatic colipase II was prepared as described [10]. Samples were dissolved in 99.8% D_2O (Stohler Isotope Chemicals) and exchangeable protons in colipase were replaced by deuterons by heating at 50°C for several minutes. The pH was measured with a Radiometer pH meter with a glass-electrode. The pH was adjusted with 1 M solutions of KOD and DCl. Proton NMR spectra were recorded using a Bruker WH-270 FT NMR spectrometer with a Nicolet 1085 computer. Chemical shifts are quoted in ppm downfield from DSS (2,2-dimethyl-2-silapentane-5-sulphonate).

3. Results and discussion

The NMR spectrum of colipase was presented [11]. The colipase molecule studied in that investigation contained 84 amino acid residues. The colipase molecule studied in the present investigation contained 5 additional amino acid residues in the N-terminal

and 11 in the C-terminal. This difference in amino acid composition does not seem to have any major effect on the colipase NMR spectrum, although differences in the chemical shift values of some resonances in the aromatic region are observed.

3.1. Histidine titrations

The aromatic region (δ_{DSS} 6–9 ppm) of the NMR spectrum of colipase at different pH values is shown in fig.1. The chemical shift of the C(2) proton resonance of histidine normally varies with pH within the range 7.7–8.7 ppm. The exact position of the resonance is dependent on the ionization state of the imidazole ring and a titration curve may be obtained when the chemical shift is plotted as a function of pH [9]. Resonance I and II show a titration behavior characteristic for C(2) protons and the pK_a values were determined by a best-fitting procedure of the curves in fig.2. The apparent pK_a value of histidine I, resonance I, was thus determined to be 7.8. We ascribe the somewhat high pK_a value to the proximity of negatively-charged groups [12]. There is a slight inflexion of the titration curve at low pH. This could be due to changes in ionization state of an acid side-chain in the proximity of histidine I having a pK_a value of about 5.2, e.g., glutamic acid which would be consistent with the observation above.

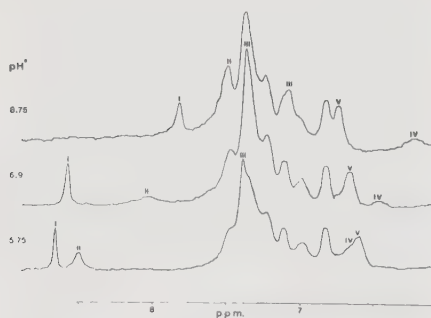


Fig.1. Aromatic region (δ_{DSS} 6–9 ppm) of colipase II. The colipase concentration was 4 mM in D_2O and 150 mM NaCl at a pH* (pH meter reading in D_2O) of 8.75, 6.9, 5.75. The resonances are labelled I–V as described in the text. 500 scans were recorded in 8 K memory with a repetition time of 0.7 s.

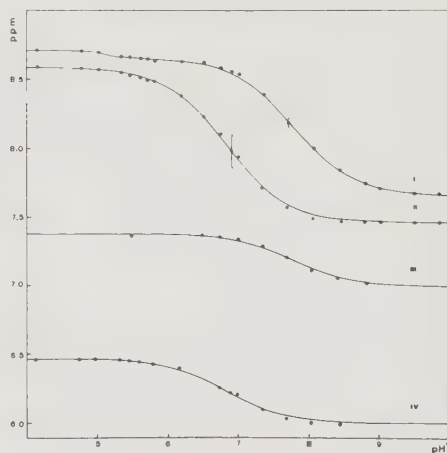


Fig.2. pH titration curve of the C(2) and C(4) proton resonances of the two histidyl residues of colipase (resonance I–IV). The brackets indicate the line-width of the resonances.

Histidine II was found to have an apparent pK_a value of 6.9. As is evident in fig.1 the C(2) proton resonance (II) broadens considerably at intermediate titration levels. This is interpreted as due to a slow protonation–deprotonation rate of the imidazole ring [13].

The C(4) proton of a histidine titrates in the same manner as the C(2) proton although at higher field and within a smaller chemical shift range, 6.9–7.5 ppm [9]. The C(4) proton resonance of histidine I, although difficult to resolve, titrates within a normal chemical-shift range. The C(4) proton of histidine II, resonance IV, on the other hand has a considerably lower chemical-shift value in the pH range studied. The simplest explanation for this upfield shift, 0.8 ppm, is that there is a ring current-induced shift arising from a nearby aromatic side-chain.

3.2. pH effects on tyrosine resonance

The resonance at 6.8 ppm and a resonance at 7.4 ppm are shown by mutual decoupling to be a pair of doublets (fig.3). These signals change chemical shift with pH with a pK_a value of ~ 10.5 and from

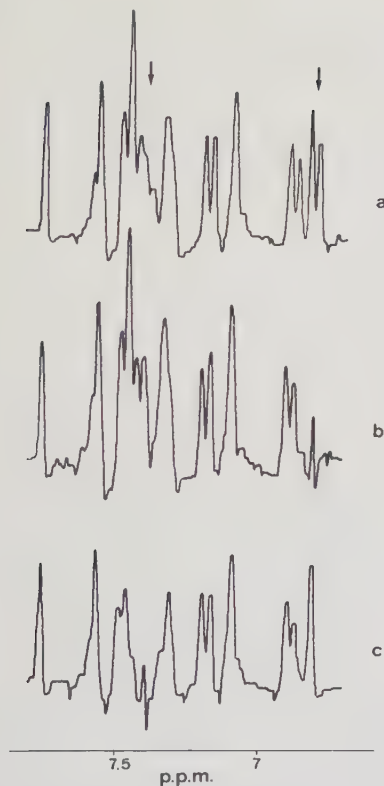


Fig.3. 270 MHz spectra of 2 mM colipase at pH* 10.0 and temp. 40°C showing the spin decoupling of the resonances of a tyrosine residue. The resolution has been enhanced by using the convolution difference technique [15]. (a) Uncoupled spectrum. (b) Irradiation at 6.8 ppm causes decoupling at 7.4 ppm. (c) Irradiation at 7.4 ppm causes decoupling at 6.8 ppm.

this information it is clear that they correspond to the *meta* and *ortho* protons of one of the three tyrosine residues in colipase. The chemical shift of the resonance from the *ortho* protons of this tyrosine also shows a strong pH dependence at low pH (fig.1). No observable shifts of the *meta* protons were noticed.

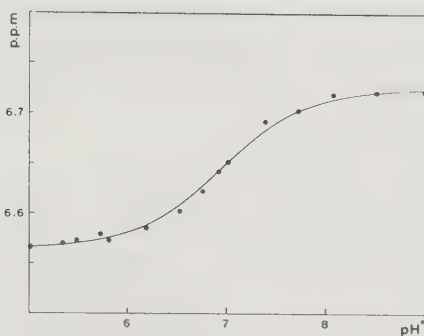


Fig.4. The pH dependence of chemical shift of the *ortho* proton resonance of one of the tyrosines in colipase (resonance V in fig.1).

If the chemical shift of the *ortho* protons is plotted against pH the resulting curve may readily be fitted to a titration curve as above (fig.4). The value of the inflexion point was calculated to be 6.9. The shift of this resonance is thus clearly correlated to the ionization state of histidine II. We interpret this upfield shift as being due to the deshielding effect of a local magnetic field within a cone on either side of the protonated histidine ring (fig.5). According to simple ring current calculations [14] the *ortho* protons of the tyrosine should be positioned within this cone at a distance from the imidazole ring of 4–6 Å. The lack of shifts of the *meta* protons suggests that the *meta* protons are positioned near an angle of 55° to the normal of the plane of the histidine ring, where the ring current field is negligible.

The upfield-shifted position of the histidine II C(4) proton resonance might be a consequence of the

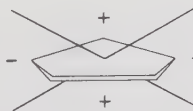


Fig.5. Schematic drawing of a protonated histidine imidazole ring with space region in which protons will experience different magnetic fields causing upfield (+) or downfield (–) shift of the NMR-signal.

ring current effect from this tyrosine, although contributions from other aromatic side-chains cannot be neglected. Whatever the case, the normal chemical-shift value of the C(2) proton could be explained in a similar manner as was done for the *meta* protons of the tyrosine residue.

3.3. *Spatial arrangement of the histidines*

It is very difficult to state from the sequence information, which of the two histidines corresponds to a specific residue in the amino acid sequence. It seems, however, likely that histidine I corresponds to His 30. This histidine has close negatively-charged groups which could account for effects on the titration curve mentioned above [12]. Furthermore, histidine II and a tyrosine must be closely related in space in order to cause the above-mentioned shifts. If the ring current effects discussed above are of mutual nature the distance between the two side-chain rings should be in the order 4–6 Å, with the planes parallel or close to parallel.

Addition of the bile salt, taurodeoxycholate, causes significant shifts and line-broadening effects on the tyrosine I and histidine II resonances. It seems likely therefore, that the suggested arrangement of these two residues forms a part of the binding area for bile salts.

Acknowledgements

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High-Resolution Proton Magnetic Resonance Study of Porcine Colipase and Its Interactions with Taurodeoxycholate[†]

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ABSTRACT: A high-resolution 270-MHz proton NMR study of porcine colipase I has been performed, and the resonances in the aromatic region of the spectrum have been assigned to amino acid residues by pH titration and decoupling experiments. The apparent pK_a values of the three tyrosines were calculated to be 10.2, 10.3, and 11.8 with one of the tyrosines having properties of a "buried" residue. A tentative assignment to the amino acid residues in the primary sequence of colipase will be discussed. The effects of taurodeoxycholate (TDC) and a positively charged deoxycholate derivative on

the aromatic region of the colipase NMR spectrum indicate that all tyrosines and one histidine are affected by the bile-salt binding, suggesting that the TDC molecules bind near these residues to a hydrophobic region on colipase. Measurements and calculations on the line width of the C(18) methyl group resonance suggest that the line-width increase of this resonance upon interaction of TDC with colipase to a large extent can be explained as due to the slower tumbling of the TDC molecules bound to colipase.

The hydrolysis of the dietary triglycerides in the intestinal lumen is mainly carried out by the enzyme pancreatic lipase with its cofactor colipase. The cofactor function of colipase is to bring the lipase molecule to the substrate surface in the presence of bile salts (Borgström & Erlanson, 1973). The

colipase molecule has been purified (Maylić et al., 1971; Erlanson & Borgström, 1972) and its primary and secondary structure characterized (Charles et al., 1974; Erlanson et al., 1974). The colipase molecule studied in the present investigation is composed of 100 amino acid residues (Figure 1). Among these, three are tyrosines, two are phenylalanines, and two are histidines. NMR¹ has proved to be a useful tool in studies of changes in the microenvironment of macromolecules upon ligand binding and in studies of the dynamics of molecular interactions (e.g., Dwek, 1973; Wütrich, 1977). In order to gain information on structural features of the colipase molecule and to interpret the interaction between colipase and TDC on the molecular level, a high-resolution proton NMR study has been performed with special reference to effects observed on the tyrosines, phenylalanines, and histidines of

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¹ Abbreviations used: CD, circular dichroism; cmc, critical micellar concentration; NMR, nuclear magnetic resonance; TDC, taurodeoxycholate; UV, ultraviolet; DSS, 2,2-dimethylsilapentane-5-sulfonate.

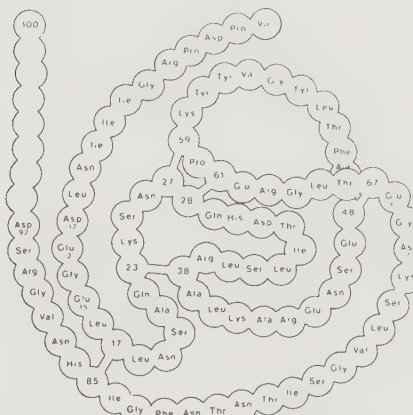


FIGURE 1: The primary sequence of porcine colipase (Charles et al., 1974; Erlanson et al., 1977).

colipase and the C(18) methyl and $-\text{CH}_2\text{SO}_3^-$ methylene group of TDC.

Experimental Section

Materials. Porcine pancreatic colipase I was purified as described (Erlanson et al., 1973) and found pure by disc electrophoresis. TDC and tritiated TDC were synthesized in the laboratory as described (Norman, 1955; Hofmann et al., 1968). The positively charged bile-salt molecule was synthesized in the laboratory. All solutions were made up in D_2O (Ciba Geigy) with a sodium chloride concentration of 150 mM. In the TDC binding studies 5 and 10 mM phosphate buffers were used. In the dialysis experiments Spectrapor 1 dialysis membranes (Spectrum Medical Industries Inc., Los Angeles) with a molecular weight cutoff of 6000–8000 were used.

Sample Preparation. Since some of the backbone-NH protons of colipase exchange slowly with the solvent D_2O , the colipase solutions were heated at 45 °C for about 15 min and cooled before spectral accumulation.

TDC titrations were performed by addition of small aliquots of a 100 mM TDC solution to the NMR tube. Colipase titrations were performed by dilution of a TDC solution of initially high colipase concentration with a similar solution containing no colipase.

NMR Measurements. Spectra (270 MHz) were run on a Bruker WH-270 NMR spectrometer equipped with a Nicolet 1085 24K computer with FT facilities. NMR tubes (5 mm) were used. The spectra were accumulated at a temperature of 30 °C. An internal deuterium lock was used. The chemical-shift values are quoted downfield from DSS. In the colipase and TDC titration experiments tetramethylammonium chloride was used as an internal reference.

pH Measurements. pH was measured with a combined glass electrode, and the samples were transferred from the NMR tube to a glass vessel before measurement. pH was varied by addition of small aliquots of 1 M KOD and 1 M DCl. All pH and pK_a values quoted are pH_{app} and pK_{app} .

Equilibrium Dialysis. The equilibrium dialysis experiment was performed in a three-chamber equilibrium dialysis cell system (Robinson & Tanford, 1975) with a Spectrapor 1 membrane between the chambers. The cells were rotated continuously during equilibration. Samples from the chambers

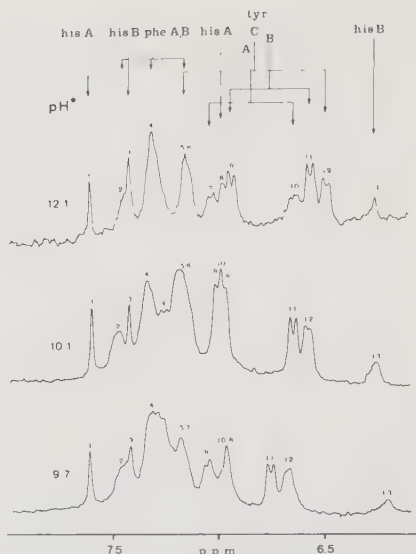


FIGURE 2: A 270-MHz proton NMR spectrum of colipase showing the aromatic region (δ_{arom} 6–9) at different pH values. The protein concentration was ≈ 2 mM in D_2O and 150 mM NaCl. The spectra are results of 500 scans. Temperature was 30 °C. The assignments are presented in Table I.

were taken every second day to follow the equilibration. TDC was allowed to dialyze from the middle chamber to a chamber containing buffer and to another chamber containing buffer and 0.15 mM colipase. Samples (50 μL) were taken and counted in a Packard scintillation counter. The equilibration continued for 7–10 days. To avoid bacterial growth all solution contained 0.02% sodium azide.

Results and Discussion

A presentation of the proton NMR spectrum of colipase and an investigation of the histidine residues in colipase have recently been reported (Cozzone, 1976; Wieloch & Falk, 1978). In the present investigation we shall assign the resonances in the aromatic region of the colipase NMR spectrum based on the pH dependence of their chemical-shift values and on decoupling experiments. Figure 2 shows the aromatic region of the colipase NMR spectrum at some different pH values. The chemical-shift values of the resonances in the spectra were plotted as a function of pH, and the data points were fitted by a least-squares procedure to the Hill equation

$$\delta_0 = \delta_A \frac{[\text{H}^+]^n}{[\text{H}^+]^n + K_a^n} + \delta_B \frac{K_a^n}{[\text{H}^+]^n + K_a^n}$$

with δ_A , δ_B , and K_a as variable parameters and $n = 1$ (Figure 3). By this procedure individual pK_a values of the three tyrosines could be obtained (Table I).

Histidines. The two histidine C(2) and C(4) protons (resonances 1, 8 and 3, 13, His-A and -B, respectively) in colipase have earlier been assigned to histidine residues with pK_a values of 6.9 and 7.8 (Wieloch & Falk, 1978). In the same investigation it was concluded that the histidine having a pK_a value of 6.9, His-B in Figure 2, is positioned near a tyrosine

Table 1: Assignment of the Resonance in the Aromatic Region of the Proton NMR Spectrum of Colipase^a

resonance	pK _a value	assignment to protons in amino acid residues	tentative assignment to residues in the structure of colipase
1	7.8	histidine C(2)	surface histidine in the proximity of negatively charged groups, possibly His-30
2	6.9	phenylalanine	a histidine in the vicinity of a tyrosine; see Figures 3 and 6; His-86
3		histidine C(2)	
4		phenylalanine	
5		phenylalanine	
6	10	tyrosine (meta)	surface tyrosine in the vicinity of a histidine (resonances 3 and 13); Tyr-56 or -57
7	11.8	tyrosine (meta)	due to the large line width of the resonance and the high pK _a value, this resonance is assigned to a buried tyrosine residue in the structure of colipase; Tyr-53
8	7.8	histidine C(4)	see resonance 1
9	10.4	tyrosine (meta)	surface tyrosine; Tyr-56 or -57
10	11.8	tyrosine (ortho)	see resonance 7
11	10.2	tyrosine (ortho)	see resonance 9
12	10.3	tyrosine (ortho)	see resonance 6
13	6.9	histidine C(4)	see resonance 3

^a The numbers in the table refer to the labeled resonances in Figure 2. The apparent pK_a values were calculated by a least-squares fitting of the chemical-shift values.

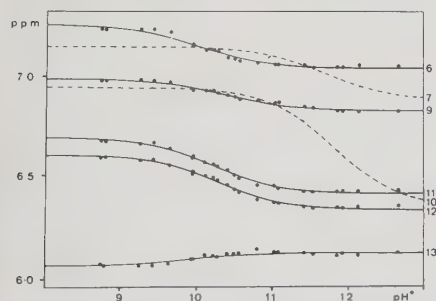


FIGURE 3: The chemical shift of the tyrosine ortho and meta proton resonances and a C(4) proton resonance of a histidine of colipase as a function of pH. The chemical-shift values of the tyrosine represent the midpoint of the resonances. The pK_a values were calculated by use of a least-squares fit to the data points as described in the text. The curves are labeled as indicated in Figure 2.

residue, Tyr-C (resonances 6 and 12). As is seen in Figure 3 the His-B C(4) proton, resonance 13, moves downfield in the pH range 9.5–10.5, which would be expected in view of the proximity of this residue to the Tyr-C residue. The assignment of the two histidines to residues in the primary sequence of colipase is in progress. A tentative assignment has earlier been made assigning His-A to His-30 and His-B to His-86 (Wieloch & Falk, 1978).

Tyrosines. In the present investigation we have determined the pK_a values of the tyrosine residues by a fit of the chemical-shift values of the ortho proton resonances. Thus the pK_a value of Tyr-C was found to be 10.3. We also observe a slight increase in the line widths of the Tyr-C resonances at pH values around the pK_a, indicative of a slower proton exchange than normally observed for free tyrosine molecules.

Resonances 9 and 11 are assigned to a second tyrosine residue, Tyr-B (Figure 2). The pK_a value was calculated to be 10.2 based on the chemical-shift changes of the ortho proton resonance. The area of each peak corresponds to two protons. Figure 4 shows the decoupling of Tyr-B resonances as a pair

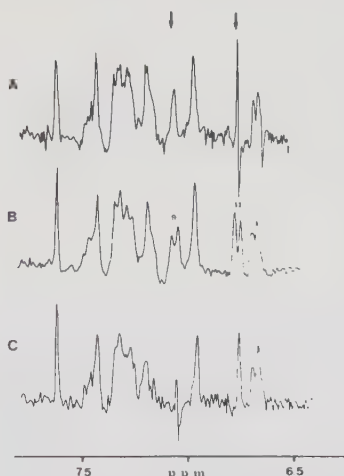


FIGURE 4: The decoupling of the two Tyr-B doublets. (A) Irradiation at 6.74 ppm causes decoupling at 7.05 ppm. (B) Undecoupled spectrum. (C) Decoupling at 7.05 ppm causes decoupling at 6.74 ppm. The resolution is enhanced by use of the convolution difference method (Campbell et al., 1973).

of mutually collapsing doublets. The splitting of the resonances is of the order of 8 Hz. The resonances are well resolved and the pK_a value is that normally found for a tyrosine residue. These findings indicate that resonances 9 and 11 arise from a freely rotating tyrosine residue probably on the surface of colipase. Resonances 7 and 10 are assigned to the third tyrosine, Tyr-A. The pK_a value was found to be 11.8, an unusually high pK_a value. The difference in chemical shift of the ortho protons of the protonated and deprotonated Tyr-A was found to be 0.57 ppm, which should be compared to 0.27 ppm for Tyr-B and -C. A conformational change of colipase accompanying the ionization of this tyrosine, possibly arising

from the disruption of a hydrogen bond to, for example, a lysine, might be the explanation of this effect. Another feature of this tyrosine is the large line width of the resonances, which indicates that the aromatic ring rotates slowly, on the NMR scale, around the $\text{C}_\beta\text{-C}_\gamma$ bond (e.g., Wütrich & Wagner, 1975; Campbell et al., 1976). In view of other investigations of tyrosines in proteins we assign these resonances to a tyrosine residue "buried" in the structure of colipase. The data from the present investigation are not sufficient to unambiguously assign the tyrosine resonances to specific amino acid residues in colipase. A tentative assignment can, however, be made by examining the primary structure of colipase (Figure 1). The three tyrosines are positioned within a pentapeptide, amino acids 53–57, of an amino acid sequence of colipase, which can be characterized as increasingly hydrophobic toward the N-terminal end and would thus be expected to be buried in the protein.

Cys-Ala-Phe-Thr-Leu-Tyr-Gly-Val-Tyr-Tyr-Lys
50 55

In view of the mobility, pK_a values, and proton exchange the following tentative assignment can be made

Tyr-A = Tyr-53 Tyr-B, Tyr-C = Tyr-56, Tyr-57

Phenylalanines. The nontitrating amino acid groups exhibiting NMR resonances in the aromatic region are phenylalanines, tryptophans, and nonexchangeable amide and hydroxyl protons. Colipase lacks tryptophan and we can assume that all backbone protons exchange with deuterons. The remaining resonances, 2, 4 (4'), and 5, can thus be attributed to the 10 protons of the two phenylalanines. Due to the large line width and thus lack of fine structure, decoupling experiments were unsuccessful, and assignments to the individual phenylalanines could not be made. In the pH range 10–12, however, small shifts of resonances 2 and 4' indicate that a conformational change might accompany the deprotonation of the tyrosines and/or the lysines.

A summary of the assignments, including a tentative assignment to specific residues in the primary structure of colipase, is presented in Table I. The assignment of resonances to specific amino acid residues in the primary structure in colipase is in progress by use of chemical modification and proteolytic degradation procedures.

Interaction with Bile Salts. Although the binding of TDC to colipase probably has no physiological importance (Donnér, 1977), it is still interesting from other points of view. By a close NMR study of the effects of TDC binding to colipase as seen in the colipase NMR spectrum, some information concerning the structural arrangement of the amino acids can be obtained. Also, it has been shown that bile salt decreases the binding between lipase (Donnér et al., 1976; Patton et al., 1978) and colipase, suggesting a competition between bile salts and lipase for the same binding site on colipase. An identification of the bile-salt binding area on colipase can thus indicate a possible lipase binding site.

The interactions between colipase and TDC have been studied by various methods. Equilibrium dialysis, gel filtration (Borgström & Donnér, 1975), and ultracentrifugation studies (Charles et al., 1975b) have shown that TDC binds to colipase at concentrations near the cmc of TDC and that the binding numbers were close to those of TDC micelles. UV spectroscopy (Charles et al., 1975a) and circular dichroism (Donnér et al., 1976) have been used to show that the aromatic residues of colipase are involved in the binding of TDC. The CD studies (Donnér et al., 1976) also showed that no major conformational changes of colipase take place when TDC is bound. It has been suggested that the TDC–colipase inter-

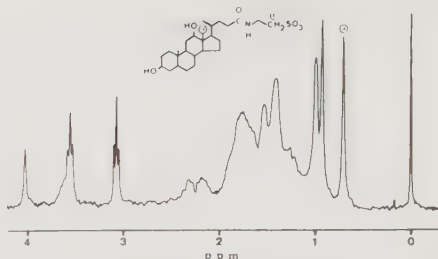


FIGURE 5: A 270-MHz proton NMR spectrum of TDC. The two resonances studied are (a) the angular methyl group (Small et al., 1969) and (b) the $-\text{CH}_2\text{SO}_3^-$ methylene group (assigned from chemical-shift tables).

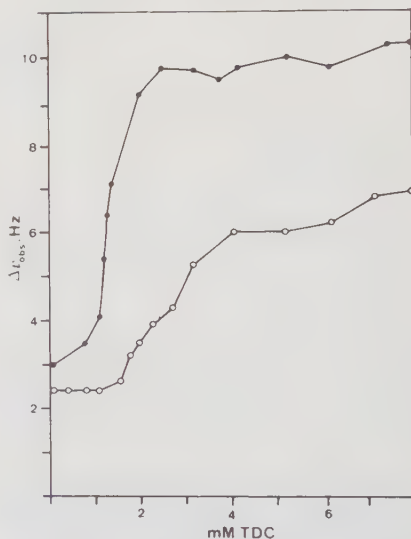


FIGURE 6: The line width of the angular C(18) methyl group as a function of TDC concentration in the absence (O) and presence (●) of 0.15 mM colipase. Phosphate buffer (10 mM), pH 7.0, 150 mM NaCl in D_2O . Temperature was 30 °C.

action is comparable to bile-salt micelle formation (Borgström & Donnér, 1976).

Observed Effects in the TDC Proton NMR Spectrum. We have studied this interaction by investigating the effects observed in the TDC and colipase proton NMR spectra. Thus Figure 5 shows a proton NMR spectrum of TDC. The resonances studied are the angular C(18) methyl group and the $-\text{CH}_2\text{SO}_3^-$ methylene group.

Figure 6 shows the line width of the C(18) methyl groups in the presence and absence of 0.15 mM colipase. The steep increase in line width at about 1 mM clearly indicates that binding of TDC to colipase takes place. The increase in line width in the absence of colipase is a manifestation of TDC micelle formation.

As the line shape of the resonances was found to be Lorentzian and as the line width increases continuously, we assume that we are confronted with a fast-exchange situation. Thus

Table II: Equilibrium Dialysis Data of the Binding of TDC to Colipase in a 10 mM Phosphate Buffer, pH 6.8, and 150 mM NaCl, Using a Three-Chamber Dialysis Cell^a

TDC _t (mM)	fraction bound (%)
0.9	4
1.1	7
1.5	22
2.2	25
3.0	26

^a The middle chamber contained a pure TDC solution; the other two contained a 0.15 mM colipase and a buffer solution, respectively.

the observed line width $\Delta\nu_{\text{obsd}}$ of the resonance is a weighted average of the line width in the bound $\Delta\nu_b$ and in the free state $\Delta\nu_f$

$$\Delta\nu_{\text{obsd}} = P_f \Delta\nu_f + P_b \Delta\nu_b \quad (1)$$

where P_f and P_b are fraction-free and -bound TDC, respectively. By use of values of P_b and P_f obtained from equilibrium dialysis (Table II) and by use of $\Delta\nu_{\text{obsd}}$ and $\Delta\nu_f$ values for the methyl signal in Figure 6, $\Delta\nu_b$ was calculated to be of the order of 25 Hz, for a concentration range of 0–3 mM TDC. By assuming the radius of colipase to be 17 Å (Erlanson & Borgström, 1972) and by using the theory of Woessner (Woessner, 1962; Navon & Lanir, 1972), the line width of the bound TDC C(18) methyl group was calculated to be 5 Hz, assuming the internal motion of the methyl group to be essentially free.

The differences in line width of 20 Hz in comparison to the experimental value of 25 Hz can be explained either as an additional increase in the rotational correlation time τ_c of the TDC–colipase complex or by dipolar interactions between the methyl protons and the protons on colipase. The former explanation, however, most likely makes the largest contribution the difference in $\Delta\nu_b$. The molecular weight of the colipase–TDC complex is of the order of 20 000 (Charles et al., 1975a), twice that of colipase, and consequently the larger τ_c of bound TDC should have a considerable effect on $\Delta\nu_b$. An additional effect is cross correlation between the pairs of interacting spins in the bound TDC methyl group (Werbelow & Marshall, 1973; Kalk & Berendsen, 1976). However, a full theoretical analysis of these latter effects in an exchanging system as is the case here is beyond the scope of this paper.

In order to study the TDC–colipase aggregates at low TDC/colipase ratios, solutions of 5 and 10 mM TDC at pH 7.0 and 150 mM NaCl and with initially high colipase concentration were stepwise diluted with solutions containing similar concentrations of TDC. The changes of the line width of the $-\text{CH}_2\text{SO}_3^-$ proton resonance were measured and plotted as $\Delta\nu_{\text{obsd}}$ vs. colipase concentration (Figure 7). In an ultracentrifugation study performed at 4 mM TDC and with varying concentrations of colipase, it was observed that large TDC–colipase complexes (Charles et al., 1975b) including more than one colipase molecule were formed. The steep increase of $\Delta\nu_{\text{obsd}}$ of the $-\text{CH}_2\text{SO}_3^-$ methylene proton resonance in Figure 7 at high colipase concentration might thus be an effect of the formation of these large aggregates, and the latter is probably also an explanation of the general broadening of all resonances in the aromatic region of the colipase spectrum, observed in the presence of TDC at pH values below 9 (Figure 9). It is thus important to choose the right pH for the TDC–colipase experiments where the TDC/colipase ratio is less than ≈ 10 and the TDC concentration is above the cmc.

Effects Observed in the Aromatic Region. In order to study the binding effects of TDC on the aromatic region of the

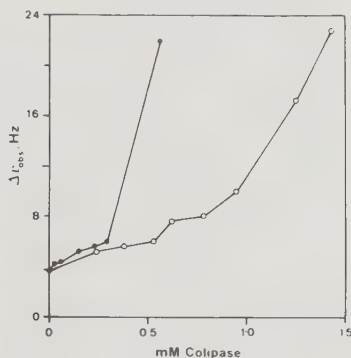


FIGURE 7: The line width of the $-\text{CH}_2\text{SO}_3^-$ methylene group as a function of colipase concentration at 5 mM TDC (●) and 10 mM TDC (○). Experimental conditions are as in Figure 6.

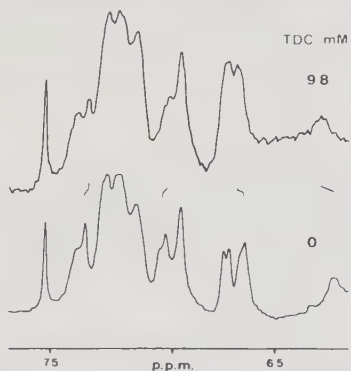


FIGURE 8: The aromatic region of the colipase proton NMR spectrum in the absence (lower) and presence (upper) of 9.8 mM TDC. The solution was made up in a 10 mM phosphate buffer, pH 9.8, 150 mM NaCl in D_2O . Temperature was 30 °C. The spectra are results of 1000 scans.

colipase proton NMR spectrum we performed the experiments at pH 9.8 and 150 mM NaCl.

Figure 8 shows the aromatic region of the colipase NMR spectrum in the absence and presence of 8.7 mM TDC.

The addition of TDC causes shifts of the His-B and Tyr-B meta and Tyr-C ortho proton resonances. The effects of the Tyr-A resonances, if any, are not detectable due to overlapping of other resonances. No shifts of the His-A and the phenylalanine residue resonances were observed.

The observed shifts of the Tyr-C and -B resonance can be interpreted as a conformational change of colipase caused by the binding of TDC or an interaction of TDC with the residues corresponding to the perturbed resonances. CD measurements (Donnér et al., 1976), however, did not show any gross conformational changes though shifts in the aromatic region were observed. UV spectroscopic studies (Charles et al., 1975a) also indicate that tyrosine residues are affected by the TDC binding. The observed effects on the tyrosine resonances in the present investigation would thus seem to indicate a direct TDC interaction with the tyrosine residues, possibly involving a small local conformational change. This is further confirmed

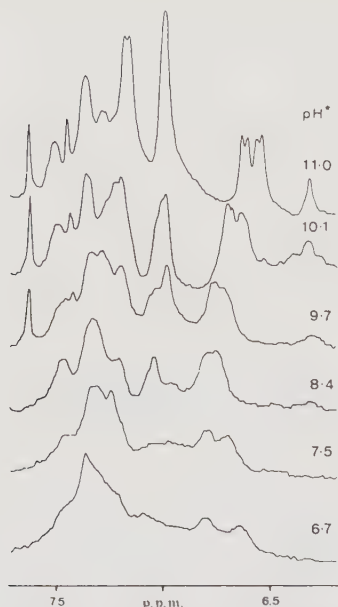


FIGURE 9: The aromatic region of colipase at different pH values and at a TDC concentration of 8.7 mM. The solutions were made up in a 150 mM NaCl solution in D₂O. Temperature was 30 °C.

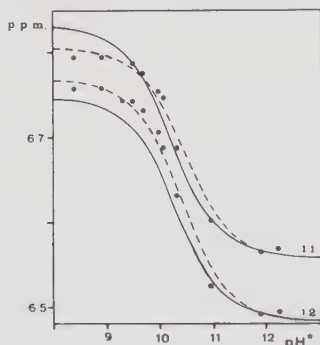


FIGURE 10: The chemical-shift values of the ortho proton resonances of Tyr-B and Tyr-C (resonances 11 and 12 in Figure 2). The solid graph corresponds to the least-squares fit to resonances 11 and 12 in Figure 3. The dashed graph is a fit to the data points obtained in the presence of 8.7 mM TDC.

by a study of the effects of pH (Figures 9 and 10).

At pH 8.5 and in the presence of 8.7 mM TDC the ortho proton resonances of Tyr-B and -C are shifted 0.09 ppm upfield and 0.04 ppm downfield, respectively, compared to the chemical-shift values in the absence of TDC. As the pH is raised the resonances are shifted upfield due to deprotonation of the tyrosine hydroxyls. However, as the pH approaches the pK_a value(s) of the tyrosine(s) the chemical shift approaches the values observed in the absence of TDC, and these are essentially equal at pH values above 11. The signals also

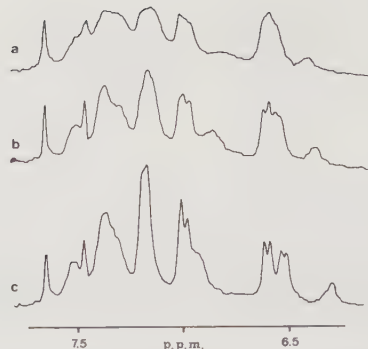
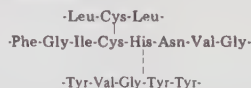


FIGURE 11: The aromatic region of the colipase proton NMR spectrum in the presence of (a) 8 mM bile-salt cation (see text), (b) 5.9 mM bile-salt cation, and (c) a pure colipase solution at pH 11.5; 2 mM colipase solution in D₂O with 150 mM NaCl.

become better resolved, indicating that no large fraction of TDC could be bound. The mutual effects observed on Tyr-B and -C and His-B resonances indicate that these three residues are closely arranged in space and thus support the tentative assignment of Tyr-B and -C to Tyr-56 and Tyr-57. The observed decrease in binding at high pH is most probably due to the repulsion between the tyrosine anions and the sulfate groups of TDC. To study this effect a positively charged deoxycholate derivative was synthesized, where the $-C(=O)NHCH_2CH_2SO_3^-$ sequence (Figure 5) was replaced by a trimethylamino group, $-CH_2N^+(CH_3)_3$. Figure 11 shows the effects of an increasing concentration of this positive bile-salt molecule on the aromatic region at pH 11.5. The Tyr-A signal shifts upfield 0.2 ppm and a general broadening of the spectrum takes place. The Tyr-B, Tyr-C, and His-B resonances also shift. The repulsive effects between the tyrosine-56 and -57 anions and the sulfate groups of TDC suggest that the observed shifts of the NMR resonances of these residues are due to the interaction with the TDC side chain, possibly involving hydrogen bondings to the tyrosines, while the steroid nucleus of TDC most probably would interact with other residues on colipase by hydrophobic interactions. In view of the earlier discussion on the structural features of colipase, such TDC binding region could have the following tentative arrangement



As was stated earlier the bile salts seem to compete with lipase for the same site on colipase (Donner et al., 1976; Patton et al., 1978). The region of colipase described above might thus be a part of the lipase binding site. Further investigations on the latter interaction are in progress using proton and carbon NMR methods.

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IS THERE A HYDROPHOBIC AROMATIC DOMAIN ON
PANCREATIC COLIPASE

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1. A 270 MHz proton magnetic resonance investigation of horse colipase B is reported and compared to earlier reported nmr-investigations of pig colipase.
2. The histidine and the tyrosine aromatic resonances were identified by pH titration and decoupling experiments.
3. The ionization of the only histidine (His-29) of horse colipase (pK_a value 6.2) induced a shift of one of the tyrosine aromatic resonances. The ionization of this tyrosine (pH^* 11-12) causes a shift of many aromatic resonances.
4. The present data shows structural similarities between the hydrophobic tyrosine rich region of horse and pig colipase as well as the proximity of one histidine to this region. This histidine is reassigned to His-30 in the structure of pig colipase.
5. The present data do not support the proposed proximity of the sequences Tyr₅₃-Tyr₅₇ and Phe₈₂-Asn₈₇ in the structure of colipase as reported earlier.
6. The bile salt-oleic acid micelles bind to the tyrosine rich region of horse and pig colipase in a similar manner as pure bile salt micelles.

Abbreviations used: DSS - 2,2-dimethylsilapentane-5-sulfonate;
nmr - nuclear magnetic resonance; FT - fourier transform;
TDC - taurodeoxycholate.

INTRODUCTION

The presence of tensioactive substances like bile salts effectively hinders pancreatic lipase to reach its triglyceride substrate *in vitro*. Pancreatic colipase secreted by the pancreas has specific binding properties to the bile salt covered lipase substrate thereby acting as an anchor on the glyceride surface for the lipase molecule (1,2).

The interaction between colipase and different amphiphilic molecules has been subject to intensive investigations using various methods (1,2). ^1H -nmr investigations of pig colipase have tentatively suggested that a hydrophobic aromatic domain on pig colipase is formed by, among others, a mutual interaction of histidine 86 and the surface tyrosine residues. This domain would constitute a general lipid binding domain on colipase (3-7). In the present proton nmr study of horse and pig colipase, the histidine resonances of these molecules are compared in order to assign them to specific positions in the amino acid sequence and to investigate the existence of a hydrophobic-aromatic domain in colipase.

MATERIALS AND METHODS

Protein preparation

Horse colipase B was purified as described (8). The protein was treated with cyanogen bromide after reduction and the three peptides analyzed for amino acid composition. The only histidine was found in the CNBr fragment I (Asn_{19} - Met_{80}) containing 62 amino acid residues (8). Porcine colipase was prepared as described (9). TDC was synthesized in the laboratory (10). Oleic acid was purchased from British Drug House. D_2O was obtained from Stohler, Switzerland and DCl and NaOD from Ciba-Geigy.

NMR measurements

The proton nmr spectra were recorded on a Bruker WH-270 nmr spectrometer equipped with FT facilities. The protein solutions were usually 1-2 mM and first heated in D₂O for 15 min at 45°C in order to exchange backbone -NH and -OH protons with deuterons. 500-2000 scans were usually collected to obtain a nmr spectrum. When possible pK_a^- values were calculated using the Hill equation. The pH and pK_a^- values quoted are pH_{app} and $pK_{a_{app}}$. As internal reference the HDO resonance was used but all chemical shift values are quoted downfield from DSS.

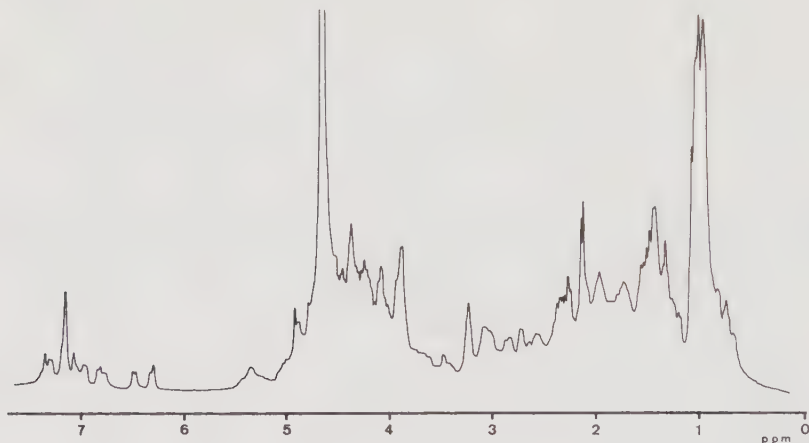


Fig. 1. A 270 MHz ¹H-nmr spectrum of horse colipase B at pH 11.2 and 40°C. 2 mM colipase in D₂O and 150 mM NaCl.

RESULTS

A ¹H-nmr spectrum of horse colipase B is shown in Figure 1. In the high field portion of the spectrum the two methionines are recognized at 2.13 and 2.09 ppm. The low field portion of the spectrum (6.0-9.0 ppm) is composed of the resonances of the aromatic protons of the three tyrosines, the two phenylalanines, the tryptophan and the C₂ and C₄ protons of the histidine (Figure 3a).

The pH dependence of the aromatic resonances

The pH titration curves of the resonances, as labelled in Figure 3a, are shown in Figure 4. From the pH dependence of the chemical shift of resonances 1 and 10 a pK_a^- value of 6.2 is calculated. The singlet fine structure of these resonances as well as their chemical shift range (Table I) strongly suggests that these resonances correspond to the C_2 and C_4 protons of the imidazole ring of the only histidine in horse colipase.

TABLE I

pK_a^- values and the range of the chemical shift between the protonated and deprotonated forms of the histidine residues in horse colipase B and pig colipases as measured by proton nmr and pH titrations.

Colipase	Resonance	pK_a^- value	δ_{H^+}	δ_{OH^-}
Horse	His-29 C_2	6.2	8.70	7.57
	C_4		6.78	6.38
Pig	His-A C_2	7.8	8.74	7.70
	C_4		7.40	7.00
	His-B C_2	6.9	8.67	7.52
	C_4		6.70	6.30

In order to assign the resonances of the tyrosines the methods used in earlier investigations (3,4) were adopted. Thus, resonances 7 and 8 were found to mutually collapse upon irradiation during decoupling experiments. These resonances titrated with a pK_a^- value of 10.4. Similar behaviour was observed with resonances 5 and 11. Resonance 11 titrated with a pK_a^- value of 10.2. Resonances 5 and 11 and resonances 7 and 8 are thus, assigned to two of the three tyrosine residues of colipase, respectively. As the linewidth was found to be in the same range as is usually found in freely rotating tyrosines and the pK_a^- values are in the range expected for tyrosines, we assign these resonances to two surface tyrosine

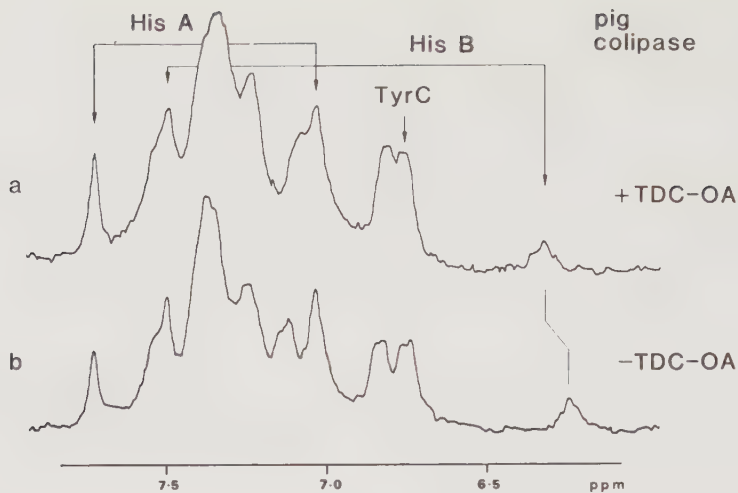


Fig. 2. The aromatic region of pig colipase at pH 9.3 in the presence (a) and in the absence (b) of 20 mM TDC and 4 mM oleic acid. 2 mM colipase in D_2O and 150 mM NaCl, 40°C.

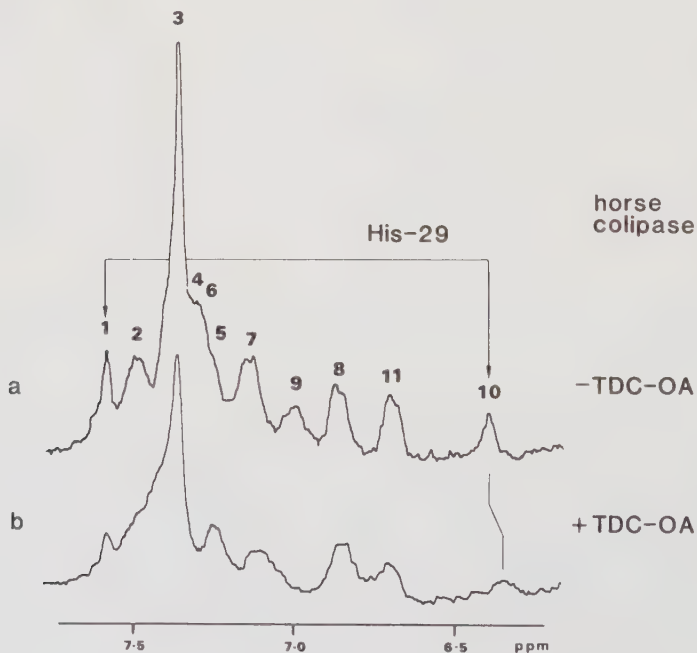


Fig. 3. The aromatic region of horse colipase B at pH 9.3 in the absence (a) and in the presence (b) of 20 mM TDC and 4 mM oleic acid. 2 mM colipase in D_2O and 150 mM NaCl, 40°C.

residues. Resonances 6 and 9 are assigned to a tyrosine residue buried in the protein structure. These resonances titrate with an anomalous pH dependence characterized by a narrow pH range (1 pH unit) and a large chemical shift difference between the protonated and deprotonated form. These resonances are considerably broader as compared to resonances 5, 7, 8 and 11 indicative of a slow rotation of the aromatic ring on the nmr time scale. The three tyrosines of pig colipase were found to have similar characteristics (3,4). Resonances 2, 3 and 4 are finally assigned to the two phenylalanines and the tryptophan. Due to the lack of resolution at this magnetic field these resonances could not be individually assigned.

Comparison between the aromatic region of the nmr spectrum of horse and pig colipase

We have earlier reported nmr-investigations of porcine colipase (3,4). The low field region of the nmr-spectrum of this protein is shown in Figure 2b. Pig colipase has a similar amino acid composition as horse colipase but lacks a tryptophan and methionines but has two histidines labelled His-A and His-B in Figure 2a. The pH dependence of the C_2 and C_4 proton resonances of His-A and His-B and the Tyr-C ortho-proton resonance is shown in Figure 4. Table I shows the pK_a values of the histidine residue of horse and pig colipase. It should be noted that the horse histidine residue has a lower pK_a value, 6.2, as compared to any of the two histidines in pig colipase which were found to be 6.9 and 7.8. The chemical shift range, however, of the resonances of His-29 in horse colipase and His-B in pig colipase are similar, specially that of the C_4 proton resonance. In addition, as the horse histidine residue is ionized, a shift is observed of the ortho resonance of the "buried" tyrosine residue (resonance 9). A small chemical shift change was observed on resonance 11. In pig colipase similar effects were observed, although in this case mutual chemical shift changes of the C_4 resonance of His-B and a surface tyrosine, Tyr-C, ortho resonance were observed. The second histidine, His-A, of pig colipase is not affected by the ionization of the tyrosine residues and vice versa (3,4).

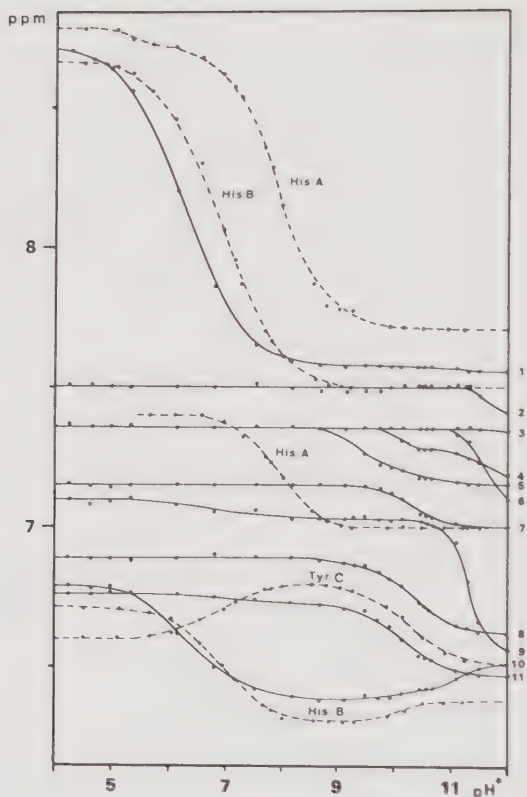


Fig. 4. The pH dependence of the proton resonances in the aromatic region of horse colipase B (—) and pig colipase (----). The curves are labelled as described in Figures 2 and 3.

Effects of TDC-oleic acid micelles

The effects of TDC-oleic acid micelles on the aromatic region of pig and horse colipase is shown in Figure 2a and 3b, respectively. In horse colipase chemical shift changes are observed on the histidine C_4 and the tyrosine resonances. In the pig colipase spectrum substantial effects are observed on the His-B C_4 and the surface tyrosine resonances. The His-A resonances of pig colipase are not affected.

DISCUSSION

A hydrophobic aromatic domain on pig colipase has been proposed to be formed by the mutual interaction of His-86 and a surface tyrosine residue. The basis of this proposal was a tentative assignment of the four histidine resonances in the proton nmr-spectrum of pig colipase to specific amino acid residues in the protein (3-7). A powerful strategy used in the assignment of proton nmr-resonances to specific protons in the protein, however, is to compare the nmr-spectrum of proteins from different animal species having small differences in amino acid composition, and hopefully in the residues to be assigned (11).

TABLE II

The primary sequence of horse colipase B (8) and pig colipase (9). The histidine and tyrosine residues are enclosed in boxes.

	1	5	10	15	20	25	30																												
Pig	V	P	D	P	R	G	I	I	I	N	L	D	E	G	E	L	C	L	N	S	A	Q	C	K	S	N	C	C	Q	H	D	T	I	L	
Horse	:	:	:	:	:	V	:	:	:	:	E	A	:	:	I	:	M	:	:	:	:	:	:	:	:	E	:	:	H	R	E	S	:	:	
	35		40		45		50		55		60		65																						
	S	L	R	C	A	L	K	A	R	E	N	S	E	C	A	F	T	L	Y	G	V	Y	Y	K	C	P	C	E	R	G	L	T	C	E	
	:	:	A	:	:	A	:	:	S	:	:	:	:	S	:	W	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
	70		75		80		85		90																										
	G	D	K	S	L	V	G	S	I	T	N	T	N	F	G	I	C	H	N	V	G	R	S	A	S										
	V	:	:	T	:	:	:	:	:	M	:	:	:	:	:	:	:	F	D	A	A	:	:	E	✕	R									

The amino acid sequence of horse and pig colipase are highly homologous (Table II): The amino acid composition of colipase from different species are also extremely similar (1). All species studied sofar have three tyrosines and at least one histidine residues. Moreover colipases from different species cross activate different lipases (12). The structure of colipase thus should be extremely conserved. By comparing the low field region of proton nmr-spectra of horse and pig colipase it should thus be possible to assign the resonances of His-A and His-B of pig colipase to the specific histidines in

the sequence. Horse colipase has one histidine at position 29, while the histidines of pig colipase are at position 30 and 86, Table II.

The C_2 and C_4 protons resonances of His-B in the pig colipase nmr-spectrum has clear similarities with those of the horse colipase histidine resonances. a) The chemical shift values of the resonances are similar. b) The ionization of the both histidines induce a chemical shift change of a tyrosine resonance. c) The most important similarity is the effect of the binding of TDC-oleic acid micelles. These micelles are known to bind to colipase (13,14) thereby enhancing the binding of lipase to colipase (13). The chemical shift of the C_4 protons of His-B in pig colipase and the C_4 histidine proton of horse colipase are changed by the binding of TDC-oleic acid micelle to colipase (Figure 2 and 3). This binding is similar to that observed when pure TDC-micelles bind to colipase (4).

Neither of the properties stated above were observed on the resonances of His-A of pig colipase.

The present data thus clearly shows that His-B of pig colipase have similar nmr properties as His-29 of horse colipase. In spite of the small positional differences we are inclined to reassign His-B of pig colipase to His-30. The difference in the effects on the tyrosine residue upon ionization of these histidine residues as well as the difference in pK'_a values may well be attributed to this positional difference. The other histidine in pig colipase, His 86, is replaced by a phenylalanine in horse colipase B (Table II). Due to the overlapping of the resonances the phenylalanine resonances could not be resolved and studied. The proposed concept of a hydrophobic aromatic domain composed of the sequences Tyr₅₃-Tyr₅₇ and Phe₈₂-Asn₈₇ in pig colipase (3,7) can thus not be verified by the present investigation.

The present investigation, however, clearly shows that the tyrosine rich region is involved in the binding of TDC-oleic

acid micelles and that this binding induce similar changes in the colipase nmr spectrum as was observed in the presence of pure TDC micelles. This supports the idea that the tyrosine rich region of colipase is part of a general lipid binding site (15).

The binding constant between pancreatic lipase and colipase have been determined by microcalorimetry to 10^{-6} M (16). These studies also showed that the binding of TDC micelles to colipase decrease the binding between lipase and colipase with a factor of 10, in the absence of triglyceride substrate (16). On the other hand, gelfiltration studies have indicated that the lipase-colipase binding increases by a factor of 100 in the presence of TDC-oleic acid micelles (13). The binding constant between colipase and the TDC-oleic micelles is also larger compared to that between pure TDC micelles and colipase (14). The present data, however, shows that the structural effect of TDC-oleic acid micelles on colipase are similar to those observed when pure TDC micelles bind to colipase (4). As the effect on the lipase binding to colipase of these two micelles is opposed the observed structural change in colipase can not be of critical importance for the lipase-colipase binding.

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EVIDENCE FOR A PANCREATIC PRO-COLIPASE AND ITS ACTIVATION BY TRYPSIN

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1. Introduction

Pancreatic colipase is a polypeptide secreted into the pancreatic juice, necessary for the binding of lipase to its triglyceride substrate in the bile-containing content of the small intestine [1]. Colipase has been purified from the pancreatic glands of several species [1] and obtained with a varying number of amino acids. Colipases from porcine sources have been described with 68–70, 83–84 and 94–95 amino acid residues and N-terminal glycine [2]. The complete sequence of colipase₈₄ has been determined [3]. Colipases with 102–107 amino acids and N-terminal valine was first isolated from the porcine gland [4] and later from bovine and horse pancreas [5,6]. The N-terminal sequence of these colipases is Val–Pro–Asp–Pro–Arg–Gly–Ile–Ile–Ile– (4,5,6) (in the horse Ile is replaced by Val [5]). It appears that these later colipases are the forms produced by the glands and that the shorter ones have been formed by limited proteolysis before or during the purification. As the –Arg–Gly– bond of the intact colipase seemed to be susceptible to cleavage by trypsin we have investigated the course of the reaction taking place when porcine colipase₁₀₁ is treated with Sephadex-linked trypsin in vitro. Such treatment leads to the rapid and complete cleavage of the N-terminal pentapeptide Val–Pro–Asp–Pro–Arg leaving a colipase₉₆ with N-terminal Gly–Ile–Ile–Ile–.

The cleavage of the N-terminal pentapeptide by trypsin does not result in any significant increase of the specific activity in a tributyrin-based colipase assay system.

Colipase₉₆, however, binds to a phospholipid

stabilized triglyceride emulsion to a much higher extent than colipase₁₀₁ and is also 50–100 times more active to overcome the lag phase for triglyceride hydrolysis catalysed by lipase with such a substrate.

We propose that the colipase with N-terminal valine produced in the pancreatic gland is a *pro-colipase* that is activated by trypsin in the intestinal content. The biological importance of this activation is to produce a colipase which binds to a triglyceride interface covered by phospholipid, thereby making the triglyceride available for pancreatic lipase.

2. Material and methods

Colipase I was prepared as in [4] from a protein fraction of porcine pancreas generously supplied by Novo, Bagsvaerd. Porcine pancreatic lipase used for assaying colipase activity was prepared as in [7]. Chemicals used were of analytical grade, taurodeoxycholate was prepared in this laboratory, dextran T-500 was from Pharmacia, Uppsala and PEG-6000 was obtained from Union Carbide, New York. Intralipid (Vitrum, Stockholm) is a 20% emulsion of fractionated soy bean triglyceride stabilized by egg lecithin. Polyacrylamide gel electrophoresis was performed as in [4]. Trypsin was immobilized on Sephadex G-75 as in [8]. It had spec. act. ≈ 300 mU/mg dry gel.

Amino acid analysis was performed using a Durum D-500 Amino Acid analyser. Hydrolysis (24 h) figures were except for Val, Leu and Ile, for which 72 h hydrolysis figures were used. Serine and threonine values were given as zero-time hydrolysis values obtained by extrapolating from the 24 h and 72 h values.

N-terminal amino acids were analysed by the dansylation technique. N-terminal sequences were obtained using a Beckman 890 C Sequenator. (Kindly performed by Dr J. O. Jeppson, Department of Clinical Chemistry, Malmö). Binding of colipase to tributyrin and Intralipid was studied in a two-phase aqueous partition system as in [9].

The effect of colipase on the lag time for hydrolysis of Intralipid was determined as follows. Intralipid (0.5 ml) was diluted to 10 ml in a solution 150 mM in NaCl, 4 mM in taurodeoxycholate, 1 mM in CaCl_2 , and 2 mM in Tris-HCl (pH 8.0), at 40°C. The colipase sample was added, followed after 1 min by 10 μl porcine pancreatic lipase (10^{-5}M). The lag time passing before lipase started to hydrolyze the triglyceride of the Intralipid emulsion was measured by pH stat titration [9].

3. Results

3.1. Cleavage of colipase₁₀₁ with trypsin:

Isolation of peptides

Colipase I (10 mg) with the amino acid compo-



Fig.1. Polyacrylamide gel electrophoresis at pH 8.3 of colipase I (= colipase₁₀₁) at zero time and after incubation with Sephadex-trypsin for 30 min, 1, 2, 4, 6 and 24 h as described in the text.

sition given in table 1 were dissolved in 8 ml 0.05 M NH_4HCO_3 (pH 8.5) and added in a test tube to 3 mg pre-swollen Sephadex-linked trypsin to 10 ml total vol. Incubation took place at room temperature with stirring. At intervals from 0.5–24 h, 1 ml samples of the solution were removed for analysis after short centrifugation to separate the gel. Polyacrylamide gel electrophoresis at pH 8.3 of the samples is seen in fig.1. A new band with higher mobility than the starting material appeared after 0.5 h and an almost complete conversion was seen after 6 h. At longer incubation another weak band started to appear with a lower mobility.

To isolate the main cleavage products a preparation with 100 mg colipase₁₀₁ and 30 mg Sepharose-trypsin was run in 10 ml total vol. for 24 h. After separation of the gel the solution was lyophilized, redissolved in a small volume and filtered through a Sephadex G-25 superfine column (1.5 × 90 cm). Two peaks at A_{230} were obtained. The void volume peak which had colipase activity was applied to a QAE-Sephadex column (2.6 × 40 cm) equilibrated in 0.01 M NH_4HCO_3 (pH 8.0). After elution with 200 ml of the same buffer a linear gradient was applied (0.01–0.30 M NH_4HCO_3 (pH 8.0), 500 ml of each). One major peak was obtained which at electrophoresis at pH 8.3 showed one band appearing ahead of the original colipase₁₀₁ and corresponding to the main product in the time-related reaction. The amino acid composition of this protein is given in table 1. The N-terminal sequence was Gly-Ile-Ile-Ile- and the total number of amino acid residues 96. This protein is in the following referred to as colipase₉₆. The second peak from the Sephadex G-25 column appeared close to the total volume and contained a pentapeptide with the amino acids given in table 1 with N-terminal valine. Colipase₁₀₁ thus was rapidly and completely cleaved by trypsin between Arg₅ and Gly₆ resulting in colipase₉₆ and a pentapeptide. The latter had no colipase activity.

3.2. Binding of colipase₁₀₁ and colipase₉₆ to Intralipid and tributyrin

In a two-phase aqueous partition system [9] a weak and inconsistent binding of colipase₁₀₁ to Intralipid was found; with colipase₉₆ binding to Intralipid occurred. The dissociation constant calculated from the Scatchard plot for colipase₉₆ was $K_d = 1.0 \times$

Table 1
Amino acid composition of colipase and the products obtained by limited proteolysis

Amino acids	Number of residues					
	Colipase I		Colipase I residue		Peptide	
	Expt ^a	Nearest integer	Expt ^a	Nearest integer	Expt	Nearest integer
Ala	4.64	5	4.73	5	—	—
Arg	5.19	5	4.12	4	1.03	1
Asx	13.06	13	12.37	12	1.00	1
Cys	n.d. ^b	10	n.d. ^b	10	0.09	—
Glx	9.90	10	10.03	10	0.05	—
Gly	8.54	8–9	8.85	9	—	—
His	2.03	2	2.01	2	—	—
Ile	5.76 ^c	6	5.48 ^c	5–6	—	—
Leu	9.57 ^c	10	9.91 ^c	10	—	—
Lys	4.15	4	4.31	4	—	—
Met	—	—	—	—	—	—
Phe	2.07	2	2.20	2	—	—
Pro	3.44	3	0.74	1	2.00	2
Ser	11.80 ^d	12	10.04 ^d	10	0.18	—
Thr	5.05 ^d	5	5.23 ^d	5	—	—
Trp	—	—	—	—	—	—
Tyr	2.64	3	3.24	3	—	—
Val	3.73 ^c	4	3.01	3	0.80 ^c	1
Total no. residues	101.57	101–102	96.27	95–96	4.83	5

^a Except where noted the figures are taken from the 24 h hydrolysis value

^b Not determined

^c 72 h hydrolysis value

^d Values obtained by extrapolation to zero time hydrolysis

10^{-7} M. The substrate–water interface to which colipase adsorbs may be modified by the reagents of the two phase system. This does not, however, invalidate the observation of a difference in the binding of the two colipases.

The K_d for the binding of colipase₁₀₁ and colipase₉₆ to a tributyrin emulsion was found to be 1.5×10^{-7} M with no significant difference.

3.3. Biological activity of colipase₁₀₁ and colipase₉₆

The activity of colipase₉₆ to restore lipase activity of an emulsion of tributyrin dispersed with bile salt was only 1.2–1.5-times greater than that for colipase₁₀₁, as measured as V_{max} from a Lineweaver-Burke plot. In these experiments lipase and colipase in equimolar concentrations were incubated with increasing amounts of tributyrin emulsified in a con-

stant volume of buffer containing bile salt at pH 7.0.

When lipase was incubated with Intralipid in bile salt suspension no significant hydrolysis took place. With colipase added, triglyceride hydrolysis occurred at a high rate (dependent on lipase concentration) after a lag time. The length of the lag period was dependent inter alia on the concentration and species of colipase. Figure 2 shows a plot of the length of the lag phase for triglyceride hydrolysis versus log colipase concentration for the two colipases; parallel curves are obtained and 50–100-times higher concentration is needed of colipase₁₀₁ compared to colipase₉₆ in order to reach the same lag time. A comparison at the concentration of colipase found in pig small intestinal content, 2×10^{-7} M [9], showed a lag time for colipase₁₀₁ and colipase₉₆ of ≈ 15 min and ≈ 0.8 min, respectively.

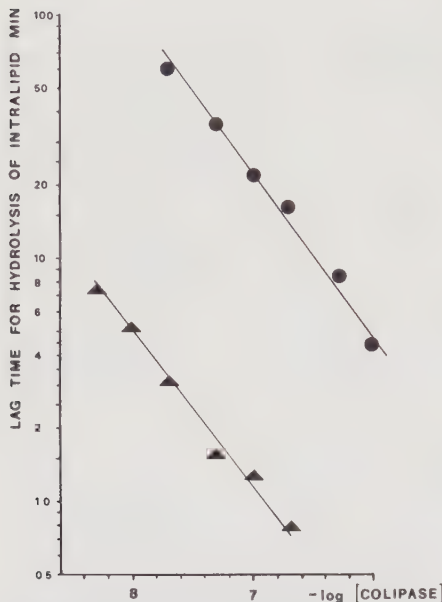


Fig.2. Effect of colipase₁₀₁ (●) and colipase₉₆ (▲) on the lag time for rapid triglyceride hydrolysis with Intralipid as substrate. Intralipid emulsion (0.5 ml) in a 10 ml total vol. buffer, 4 mM in TDC was titrated at pH 8.0 and 40°C. Colipase was added followed after 1 min by lipase (final conc. 2×10^{-6} M) and the time measured until a high rate of triglyceride hydrolysis occurred.

4. Discussion

Porcine colipase I with N-terminal valine analysed in this investigation was designed colipase₁₀₁. The present experiments show that a limited proteolysis of colipase₁₀₁ with trypsin gives colipase₉₆. Extensive treatment with trypsin causes degradation also at the C-terminal end but this has probably no biological importance.

Colipase₉₆ has a hydrophobic N-terminal sequence that has been found in the structure of colipases from all species analysed [1]. So far the only unique property recognized for colipase₉₆ is its activation of a

phospholipid covered triglyceride emulsion. This, however, may be an important biological function as dietary fat in general contains phospholipid and phospholipid is contained in appreciable quantities in the bile admixed to the small intestinal contents. The hydrophobic N-terminal tail of colipase₉₆ may interact specifically with phospholipid monolayers, as indicated by its binding to the interface of Intralipid. Such interactions are still under investigation. Preliminary experiments have revealed that no significant spectroscopic changes (NMR, ultraviolet) occur as a consequence of the conversion of colipase₁₀₁ to colipase₉₆. If colipase is synthesized and secreted as a pro-colipase, the reason might be that the activated form is incompatible with the pancreatic tissue. Perhaps the hydrophobic tail of the activated colipase would interact with the phospholipids of the intracellular membranes and disrupt the sequence of secretion.

Acknowledgements

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TRYPSIN ACTIVATION OF PORCINE PANCREATIC PROCOLIPASE

DETERMINATION OF K_m AND k_{cat}

Effects on conformation and the importance of the N-terminal
 α -amino group

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SUMMARY

Porcine procolipase₉₃ containing 93 amino acids is transformed into colipase₈₈ by the trypsin cleavage of the Arg₅-Gly₆ bond. This cleavage liberates a N-terminal pentapeptide. No cleavage in the C-terminal end or in the structure was observed. The K_m and k_{cat} values for the trypsin activation of procolipase₉₃ were calculated to 0.06 mM^{-1} and 8 sec^{-1} , respectively, at $0-1^\circ\text{C}$, pH 8.0.

Procolipase₉₃ and colipase₈₈ were reductively methylated using sodiumboronhydride and ^{13}C -formaldehyde. The methylated ϵ -amino groups of the N-terminals were studied by carbon-13 nmr. The trypsin activation changes the environment and pK_a value of the methylated α -amino groups, while the methyllysine residues were unaffected. The pK_a value of the α -amino groups were calculated to 7.4 and 7.5 for procolipase₉₃ and colipase₈₈, respectively. Proton nmr studies showed no conformational change and the pK_a values of the histidines and tyrosines were unaffected. The binding profile of colipase₈₈ to a tributyrin-bile salt interface was shifted towards basic pH values compared to procolipase₉₃. The nmr and the binding data suggests that a local change in the N-terminal region of colipase₈₈ accompanies the trypsin activation and that an ionized α -amino group is important for the interfacial binding properties of colipase and procolipase.

INTRODUCTION

Colipase is a protein participating in the duodenal degradation of dietary glycerides as a cofactor to pancreatic lipase (1,2). It has been purified by several laboratories in many forms due to its vulnerability for trypsin and carboxypeptidase attack (3,4,5). Porcine procolipase₁₀₁ purified from the pancreatic gland has a N-terminal sequence Val₁-Pro₂-Asp₃-Pro₄-Arg₅-Gly₆- (4). The Arg₅-Gly₆ is readily cleaved by trypsin (6) and the colipase₉₆ molecule thus formed were found to decrease a lag time during the lipase hydrolysis of a phospholipid stabilized triolein emulsion (6), and to have better lipid binding and lipase activating properties on negatively charged and zwitterionic interfaces at high surface pressure (7,8). It has been suggested that colipase is secreted by the pancreas in a proform in a similar manner as the pancreatic zymogens and activated by trypsin in the intestine (4). The activation of the zymogens such as trypsinogen, chymotrypsinogen (9) and phospholipase A₂ (10,11) has been thoroughly investigated as well as the effect of the activation on the molecular level (12). The present investigation compares the kinetics of the trypsin activation of procolipase₉₃ with that of the pancreatic zymogens, and investigates the effects of the activation on the structure of colipase.

MATERIALS AND METHODS

Materials

Porcine pancreatic procolipase₉₃ was purified essentially as described earlier (4). Two forms of colipase I could be separated after an additional ion exchange chromatography (SP-Sephadex pH 4.0), procolipase₁₀₁ (6) and procolipase₉₃. The amino acid compositions of the purified procolipase₉₃, its activated form and the N-terminal pentapeptide were determined on a Durrum D-500 Amino Acid Analyzer after hydrolysis during 24 and 72 hours. Lipase A was purified according to Verger (13). In order to obtain a lipase preparation free from colipase contaminations, an additional G-100 gel filtration step was

performed at pH 9.2. Bovine trypsin (DPCC treated) was obtained from Sigma. Preparative trypsin activation of procolipase was performed using Sephadex immobilized trypsin as reported earlier (6,14). TDC (15) and egg-PC (16) were synthesized in the laboratory. All other chemicals were analytical grade.

Methods

Trypsin activation: 0.6-1.4 mg procolipase₉₃ in 5 ml of a buffer solution composed of 50 mM Tris-HCl pH 8.0, 10 mM CaCl₂ and 150 mM NaCl, was cooled on an ice bath. 5 μ l of a trypsin solution (1 mg/ml) was added. Aliquots were taken at time intervals and diluted with a solution containing 10 mM DFP and 1 mg soy bean trypsin inhibitor (Sigma)/ml and colipase₈₈ activity measured. Colipase activity was measured by a pH-stat (17) as its lipase activating ability against a tributyrin dispersion in a 4 mM TDC solution at pH 8.8.

NMR: The proton nmr studies were conducted on a Bruker WH-270 spectrometer with FT facilities. Two mM solutions of (pro)colipase in D₂O, preheated in order to exchange backbone protons for deuterons, were subjected to pH titration using DCl or NaOD (Ciba-Geigy) and micelle binding studies. The ¹³C-nmr investigations were performed at 23.5 MHz on a XL-100-15 FT-spectrometer using 12 mm o.d. sample tubes and an internal deuterium lock.

Protein modification: In order to obtain ¹³C-methylated amino groups on procolipase₉₃ and colipase₈₈ the proteins were reductively methylated with sodium borohydride and formaldehyde according to the method of Means and Feeney (18) using ¹³C-enriched formaldehyde (10% water solution, Prochem, England). The procedure was performed twice in order to obtain fully methylated proteins. The colipase retained their lipase activating abilities.

Binding of procolipase and colipase to the lipase substrate: Binding studies of procolipase₉₃ and colipase₈₈ were conducted essentially as described earlier (19). 18 μ g of protein in 10 ml of a 2 mM TDC solution and 0.2 ml tributyrin was stirred

with a magnetic bar for 5 mins. pH was continuously checked during the experiment. N_2 -gas was flushed over the surface of the solution. 300 μ l of the dispersion was transferred to a tube and centrifuged in an Ole Dick microcentrifuge (Nino Laboratory, Stockholm, Sweden) for 5 s. The tributyrin-TDC dispersion sedimented but did not coalesce during this procedure. A sample was taken from the supernatant and tested for colipase activity.

Electrophoresis: Polyacrylamide electrophoresis were conducted at pH 8.3 in a gel containing 15% acrylamide (20) and in the presence of SDS in 18% acrylamide (21). The gels were fixed in a solution of 12.5% TCA and stained with 0.05% Kenacid Blue in 12.5% TCA.



Figure 1. Polyacrylamide gel electrophoresis at pH 8.3 of procolipase₉₃ after 0, 2, 5, 10, 20, 40, 90, 300 and 600 minutes incubation with trypsin gel.

RESULTS

Characterization of procolipase and colipase

The course of the trypsin-gel activation of porcine procolipase₉₃ as seen by gel electrophoresis is shown in Figure 1. Procolipase₉₃ is completely converted into colipase₈₈ after 10 hours. The product was subjected to G-25 superfine gel-filtration and the activated colipase molecule was separated from the activation products as described earlier (6). The amino acid composition of the procolipase and its activation products are shown in Table 1. The procolipase molecule contained 92-94 amino acid residues, the colipase 87-88 and the peptide 5 amino acid residues. The only bond cleaved by trypsin was the Arg₅-Gly₆ bond. No additional cleavage in the

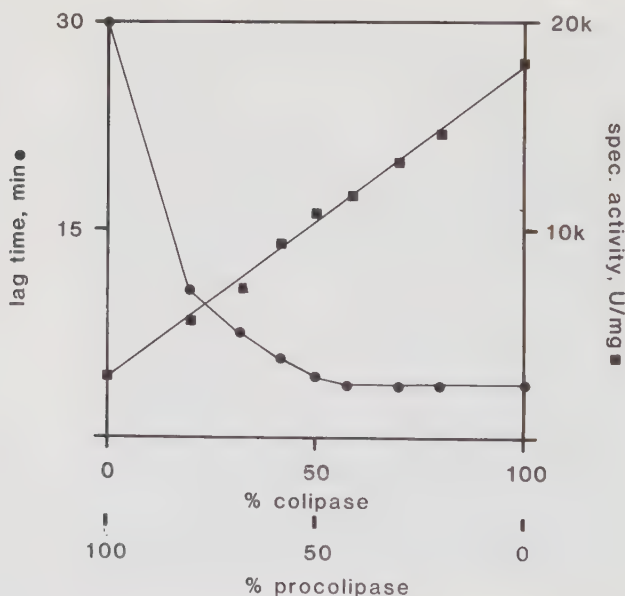


Figure 2. The specific activity (U/mg protein) and the lag time τ_d of the lipase hydrolysis of Intralipid, of different mixtures of procolipase and colipase with constant protein concentration, as a function of the ratio procolipase:colipase.

C-terminal end was observed as was reported earlier for the procolipase₁₀₁ having additional 8 amino acids in the C-terminal (6). Electrophoresis of procolipase₉₃ and colipase₈₈ in the presence of SDS and mercaptoethanol showed only one stained protein band indicating that no intramolecular cleavage takes place, for example the Arg₆₃-Gly₆₄ bond.

Kinetics of trypsin activation of procolipase

Two assay systems have earlier been used to distinguish between procolipase and colipase. The Intralipid based assay (6) and the monolayer dilaurin-TDC assay (7). In the present investigation the better binding properties to tributyrin in the presence of TDC of colipase as compared to procolipase at basic pH values have been used in order to discriminate between procolipase and colipase. At pH 8.8 the specific activity (lipase activity in the presence of bile salt and cofactor) of procolipase was 3000 U/min/mg and for colipase 18,000 U/min/mg in this assay. By mixing procolipase and colipase in different proportions and measuring the lipase activity in this assay the linearity of the method can be checked. Figure 2 shows that such a linearity exists. This assay can thus be used to study kinetics of trypsin activation. For comparative reasons the change in the lag time, τ_d , obtained during hydrolysis of Intralipid as a function of the ratio procolipase/colipase is also plotted in Figure 2.

Determination of K_m and k_{cat}

Figure 3 shows the time course of the activation of procolipase₉₃ by trypsin at 20°C. The insert stresses the first order kinetics of the activation process. The initial velocity was determined by calculating the amount procolipase transformed during the first minute of the reaction. The Lineweaver-Burk plot of the reaction is shown in Figure 4. The K_m was calculated using a least square procedure and found to be 0.06 mM⁻¹. k_{cat} was obtained using the relation $V = k_{cat}/[E_o]$ and was calculated to 8 sec⁻¹. The reaction conditions were: 0-1°C, 50 mM Tris-HCl, pH 8.0, 10 mM CaCl₂. In order to in-

TABLE I

Amino acid composition of porcine procolipase, colipase and the isolated peptide.

Amino acid	Number of residues					
	procolipase		colipase		peptide	
	expt	nearest integer	expt	nearest integer	expt	nearest integer
Ala	4.5	4- 5	4.5	4- 5		
Arg	5.0	5	3.9	4	1.0	1
Asx	11.5	11-12	10.6	11	1.0	1
Cys ^a	10	10	10	10		
Glx	9.8	10	9.2	9		
Gly	8.4	8	7.9	8		
His	1.8	2	2.0	2		
Ile ^b	5.0	5	4.9	5		
Leu ^b	8.8	9	8.9	9		
Lys	4.2	4	4.0	4		
Met	-	-	-	-		
Phe	2.0	2	2.0	2		
Pro	3.0	3	0.9	1	1.9	2
Ser	8.2	8	8.4	8	0.2	-
Thr	4.8	5	4.8	5		
Trp	-	-	-	-		
Tyr	2.6	3	2.6	3		
Val ^b	3.4	3	2.2	2	0.9	1
Total No. of residues	93.0	92-94	86.8	87-88	5.0	5

^aNot determined. Taken from (4).

^b72 h hydrolysis values

investigate whether the presence of micelles affected the trypsin activation 10 μ g of procolipase₉₃ was incubated with 10 μ g trypsin in 1 ml of solutions containing, respectively, 10 mM TDC, 10 mM TDC + 2 mM oleic acid, 10 mM TDC + 1 mM egg PC and 0.1 ml native bile in the presence and absence of 50 μ g pancreatic lipase and at 37°C. In all cases full activation was obtained after 2 mins.

Conformational effects on colipase upon trypsin activation

The conformational effects of trypsin activation of procolipase₉₃ can be studied by proton nmr. The histidine resonances and the three tyrosine resonances described earlier for procolipase (22,23) were unperturbed as well as their pK_a values (Figure 5). The mutual ionization effect of the His-B

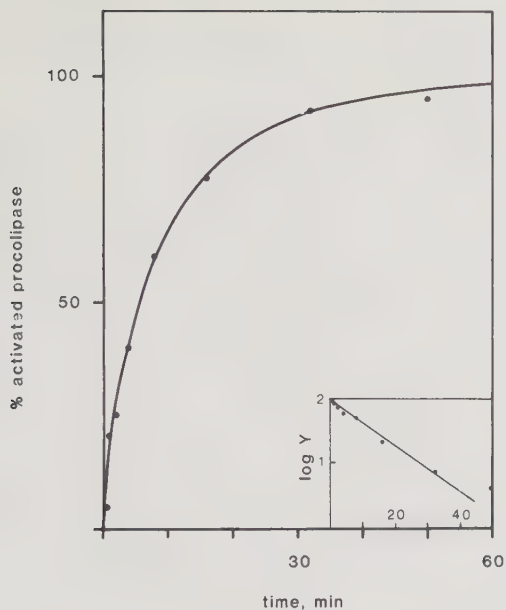


Figure 3. The activation kinetics of procolipase by trypsin. Activation was measured by following the colipase activity on the tributyrin-TDC assay at pH 8.8. Experimental conditions: Procolipase concentration 0.3 mg/ml. Trypsin concentration 0.2 $\mu\text{g/ml}$. 10 mM Tris-HCl pH 8.0, 50 mM CaCl_2 , 100 mM NaCl and 20°C . Insert: The first-order kinetics of the reaction, Y: percentage procolipase.

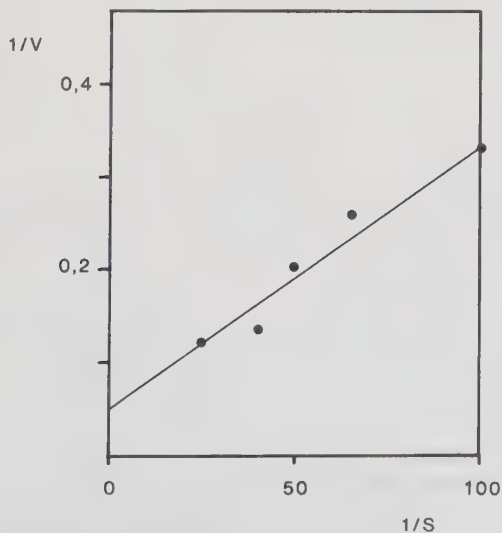


Figure 4. The Lineweaver-Burk plot of the activation of procolipase by trypsin. Experimental conditions: 10 mM Tris-HCl pH 8.0, 50 mM CaCl_2 , 100 mM NaCl, $0-1^\circ\text{C}$. Trypsin concentration 1 $\mu\text{g/ml}$. V is given as $\mu\text{moles substrat} \cdot \text{min}^{-1} \cdot \text{mg trypsin}^{-1}$. S is given as mM.

C₄ resonance and the tyrosin C ortho resonance is similar in procolipase and colipase (Figure 5).

The carbon-13-nmr spectra of methylated procolipase₉₃, colipase₈₈ and the separated pentapeptide are shown in Figures 6a-d. The pH-dependences of the chemical shift is shown in Figure 7. Reductive methylation of amino groups has earlier been applied in proton nmr in order to study the lysine residues of protein (24,25). As resonance 5 (Figure 6a) dis-

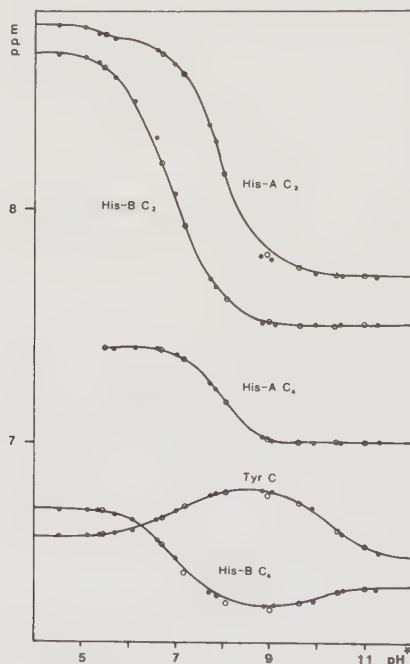


Figure 5. The pH-dependence of the proton resonances of the two histidines and one tyrosine resonance of pig procolipase (●) and colipase (○). The resonances are labelled according to (22).

appeared after trypsin activation and gel-filtration (Figure 6b) and appeared in the nmr spectra of the fraction containing the cleaved pentapeptide (Figure 6c), this resonance was assigned to the methyl group on the α -amino group of Valine-1. The pK_a value of this resonance was calculated to 7.8. The resonances 1-4 were assigned to the four methylated lysines in procolipase₉₃ with pK_a values of 9.5 (resonance 1) and 10.3 for the three overlapping resonances 2-4. Resonance 6 in

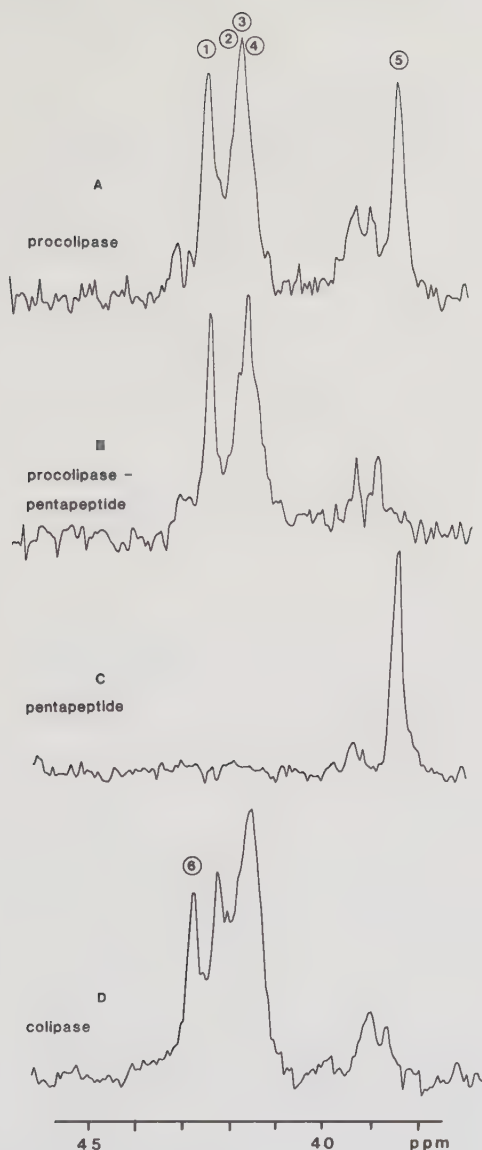


Figure 6. The ^{13}C -nmr spectra at 23.5 MHz of the enriched methyl group resonances of the methylated lysines (res. 1-4) and the N-terminal α -amino groups (res. 5 and 6) of methylated procolipase (a), methylated procolipase after trypsin activation and gel filtration (b), the cleaved pentapeptide (c) and methylated colipase (d). The chemical shift is quoted downfield from TMS. The protein concentrations were ≈ 2 mM in D_2O and 150 mM NaCl at 24°C .

the nmr spectrum of methylated colipase₈₈ (Figure 6d) is assigned to the methyl α -amino groups of glycine-6 with a pK_a value of 7.7.

The pK_a value of methylated amino groups can be transformed into values for unmodified amino groups using the correction factors obtained from free monomethyl and dimethyllysines (24). The chemical shift difference between the protonated and deprotonated forms of resonances 1-5 is 1-1.5 ppm while that of resonance 6 is 0.5 ppm. This suggests that resonance 1-5 are dimethylated and resonance 6 monomethylated and the corrected pK_a values for the different resonances would thus be 10.1 - res. 1; 10.9 - res. 2-4; 7.4 - res. 5; 7.5 - res. 6.

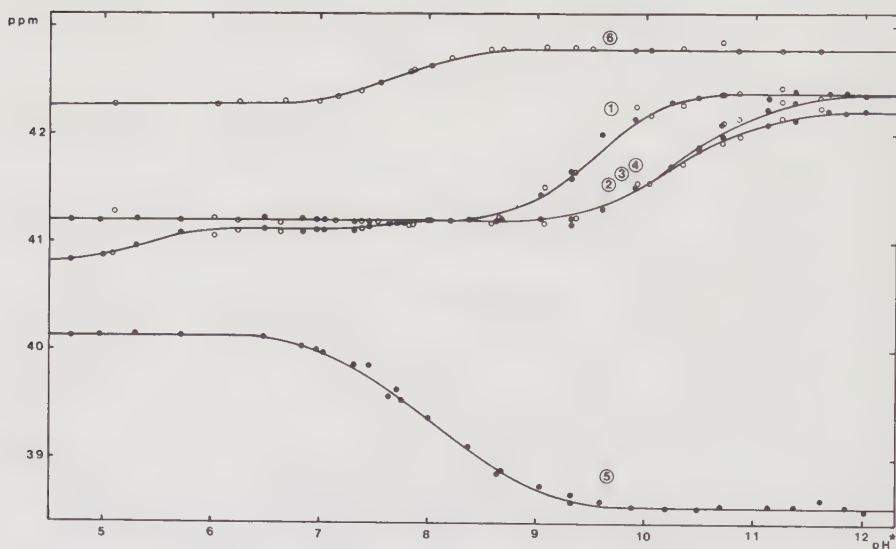


Figure 7. The pH dependence of the ^{13}C -resonances of methylated procolipase (●) and colipase (○). The resonances are labelled as shown in Figure 6.

Binding of procolipase and colipase to the tributyrin interface in the presence of 2 mM TDC

Figure 8 shows the binding profile of procolipase₉₃ and colipase₈₈ to tributyrin in the presence of 2 mM TDC as a function of pH. As is seen colipase binds better at the pH values tested. The value where 50% of the proteins are bound as compared to the initial amount bound were 7.3 and 8.1 for procolipase and colipase, respectively.

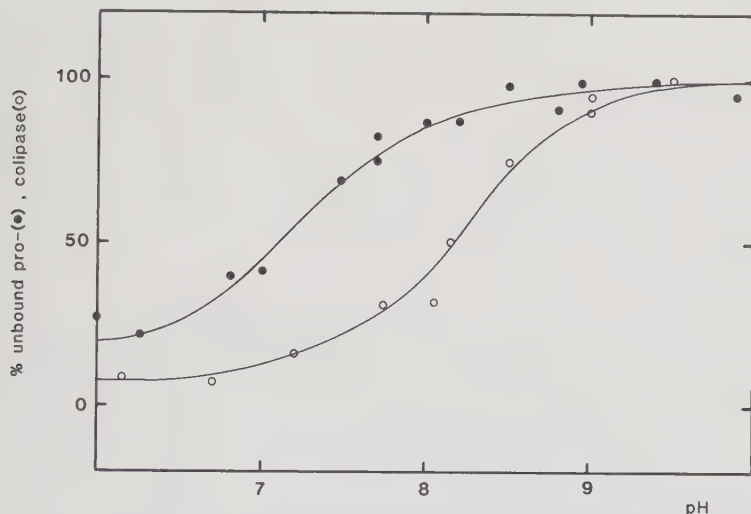


Figure 8. The pH dependence of the binding of procolipase (●) and colipase (○) to a tributyrin-TDC dispersion of 0.2 ml tributyrin in 10 ml of a 2 mM TDC, 150 mM NaCl solution. The binding was measured as the percentage procolipase free in solution as measured by a centrifugation technique (18).

DISCUSSION

In the present investigation a procolipase molecule with 93 amino acid residues was purified from a pancreatic extract obtained from a side fraction during insulin production (Novo Industries, Copenhagen). This procolipase has an intact N-terminal *i.e.* Val₁-Pro₂-Asp₃-Pro₄-Arg₅-Gly₆- but has a shorter C-terminal end compared to the procolipases reported earlier (3,4,5,6). These differences in the C-terminal, however, are of minor importance for the trypsin activation process as the longer form also are activated (6). The advantage to use pro-

colipase₉₃ in the kinetic studies of the activation process is that only one peptide bond Arg₅-Gly₆, is cleaved during activation. No intramolecular cleavage could be observed.

A similar N-terminal sequence has been reported for horse colipase. The Arg₆-Gly₇ bond in this procolipase could also be cleaved by trypsin (26). The colipases purified from man (27) and chicken (28) on the other hand have a glycine N-terminal amino acid. The N-terminal amino acid sequence was similar to the trypsin activated pig procolipase suggesting that the pentapeptide has been cleaved off during purification, although measures were taken in the latter case to avoid protease activities during the purification. The low K_m value reported here for the trypsin activation of procolipase are in the same order of magnitude as for the zymogens of pancreatic origin (9,10,11). The high k_{cat} value stresses the efficiency of the trypsin cleavage of the Arg₅-Gly₆ bond and thus its vulnerability for trypsin attack.

The activation of procolipase₉₃ results in a decreased lag time, τ_d , during lipase hydrolysis of a phospholipid stabilized triolein emulsion, Intralipid, (6). τ_d , however, does not vary linearly with the amount activated procolipase. This has been explained as accumulation of lipolytic products, fatty acid at the interface changing its properties (8). Another assay is the monolayer of rac-1,2-dilaurin systems in the presence of 2 mM TDC. In this system at high surface pressure procolipase and colipase can be discriminated.

Using tributyrin as lipase substrate in the presence of TDC the products of lipolysis are water soluble and thus does not interfere with the lipolytic process. The binding of procolipase₉₃ and colipase₈₈ to this interface was demonstrated to be dependent of an amino acid residue titrating in the range of pH 7-9 *i.e.* a basic amino acid residue. This pH dependence of the binding of procolipase is shifted to basic pH values upon trypsin activation. One effect of trypsin activation would thus be an alteration of the structure of procolipase and/or a change in the pK_a value (5) of basic amino acid residues.

The present proton nmr data do not indicate any gross conformational effects. The resonances of the histidines and tyrosines are unaffected as well as their pK'_a values. Neither the carbon-13 nmr data of the methylated lysines of procolipase₉₃ and colipase₈₈ indicate a conformational change.

The methylated α -amino group of procolipase₉₃ and colipase₈₈ on the other hand shows the largest differences. Although some of the observed chemical shift effects could be accounted for the difference in the N-terminal amino acid being methylated, the change in environment of these residues also affects the chemical shift of these resonances. It should be remained that the N-terminal sequence of colipase is highly hydrophobic, Gly₆-Ile₇-Ile₈-Ile₉-. It is thus concluded that the trypsin activation of procolipase induce a local effect in the N-terminal part of the molecule. One of these effects is a change in the ionization state of the N-terminal α -amino group. This difference is manifested as a better binding of colipase to tributyrin as compared to procolipase at basic pH values. Such a difference was also observed in a monolayer system composed of rac-1,2-dilaurin in the presence of 2 mM TDC (7). On a neutral rac-1,2-dilaurin film no difference in binding between the two forms was observed (8). The negative charges at the interfaces could thus be complementary to the positive amino groups on colipase. The change in the environment of the N-terminal amino group of colipase could thus result in a better position for interaction with a negatively charged interface. This interaction would be stabilized by the hydrophobic environment of the amino group (-ile₆-ile₇-ile₈-) and the triglyceride interface in addition to the hydrophobic tyrosine region, known to bind micelles (23). A hypothetical picture of the interaction of colipase with the negatively charged glyceride interface is shown in Figure 9.

A similar hypothesis has been put forward concerning the structure function relationship of the cardiotoxins (34). The amino groups of the lysine residues of these molecules interact with the negative charges of the phospholipids. A hydro-

TABLE I

Kinetic parameters for the activation of some pancreatic zymogens and procolipase by trypsin.

Conditions: pH 8, 1°C, 0.1 M NaCl, 50 mM CaCl₂

Zymogen	K_m (mM)	k_{cat} (S ⁻¹)
Porcine trypsinogen (9) Lys ₆ -Ile ₇	0.48	0.0055
Chymotrypsinogen (A) (9) Arg ₁₅ -Ile ₁₆	1.09	0.18
Chymotrypsinogen B (9) Arg ₁₅ -Ile ₁₆	0.57	1.6
Prophospholipase A ₂ (10) Arg ₇ -Ala ₈ (11)	2.2 0.04	7 0.36
Procolipase Arg ₅ -Gly ₆	0.06	8

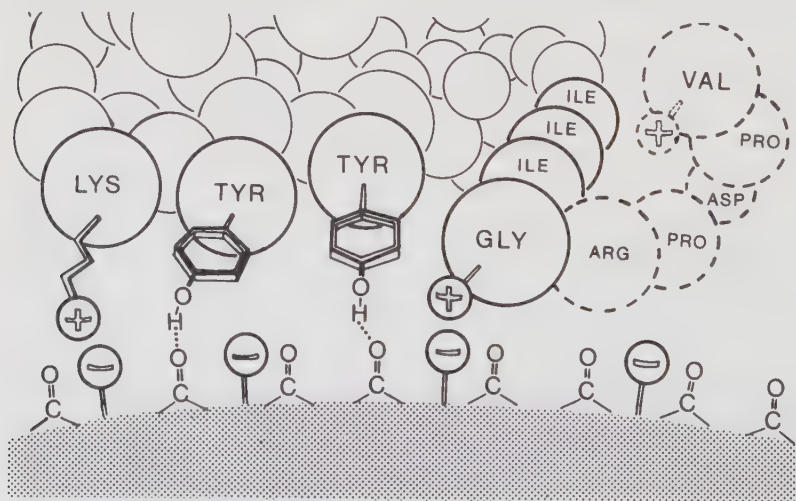


Fig. 5. A hypothetical picture of the binding of colipase to the triglyceride interface covered with charged lipids.

phobic loop then penetrates the phospholipid bilayer of the membranes.

The importance of the amino groups on PLA_2 for enzymatic activity has also been demonstrated (10). Furthermore, a negatively charged substrate interface is preferred by this enzyme (35). The crystal structure of phospholipase A_2 also reveals that hydrophobic and an excess of basic amino acid residues surrounds the active site, assumed to bind to the phospholipid interface (36).

Which is the physiological active form of porcine colipase in the intestine? It has been shown that the colipase molecule is present in the pancreas in a proform and that it is stored in the zymogen granules (29). It has also been shown that the composition of the pancreatic juice with regard to zymogens is similar as that of the zymogen granules (30). It is thus likely that colipase is secreted in the juice in a proform. Recently it was shown that lipase and colipase bind very strongly to a bile lipoprotein complex present in the bile (31). The molecules also bind other types of micelles such as bile salts (1), bile salt-fatty acid and bile salt-phospholipid (32) micelles. These micelles, however, do not affect the trypsin activation of procolipase, which thus certainly takes place in the intestinal lumen. The physiologically active porcine colipase is thus a molecule devoid of a pentapeptide and with a glycine N-terminal.

The dietary fat presented to the colipase and lipase molecule in the intestine is covered by amphiphilic molecules like bile salts, fatty acids and phospholipids. The surface presented to colipase and lipase is thus a negatively charged highly packed interface.

The present investigation have shown that the positive charge of the α -amino group of colipase is important for its binding to such interfaces.

ACKNOWLEDGEMENT

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PORCINE PANCREATIC PROCOLIPASE AND ITS TRYPSIN ACTIVATED FORM

Lipid binding and lipase activation on monomolecular films

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INTRODUCTION

Tri- and diglycerides passing into the small intestine are hydrolyzed to fatty acids and 2-monoglycerides by pancreatic lipase assisted by its protein cofactor colipase [1,2]. Both lipase and colipase are secreted by the pancreas and it has recently been shown that colipase is synthesized as a precursor form [3]. Cleavage of the N-terminal pentapeptide by trypsin yields an activated molecule. This proteolytic cleavage is assumed to take place when colipase is secreted into the duodenum in a similar way as for the zymogens of pancreatic origin.

The importance of procolipase activation was first observed in a substrate system using the commercially available Intralipid emulsion (long chain triglycerides emulsified with phospholipids) and was demonstrated as a decrease in a lag time before lipase hydrolysis proceeded at a high rate [3].

We have now studied the interactions of colipase and its pro-form with a monomolecular rac-1,2-dilaurin film in the presence of taurodeoxycholate and their lipase activating properties. In this system, at high surface pressure, we have observed an all or none effect on the lipase activation by colipase and procolipase, respectively. These results support the idea that trypsin activation of procolipase yields a colipase molecule with better lipid binding properties. This can be compared to the well documented case of trypsin activation of phospholipase A₂ [4,5]. In view of earlier results the relevance of procolipase activation to the *in vivo* situation will be discussed.

MATERIALS AND METHODS

Lipids

rac-1,2-didodecanoylglycerol (1,2-dilaurin) was obtained from Serdary Research Laboratories (Ontario, Canada) and showed one major spot by thin layer chromatography. Sodium taurodeoxy-

cholate, TDC, (SIGMA) was used with no further purification. All other chemicals were analytical grade.

Proteins

Porcine pancreatic procolipase and colipase were prepared as described earlier [6,3]. Porcine pancreatic lipase LA and LB were purified according to Verger [7], with an additional Sephadex G-100 filtration at pH 9.2 and 0.05 M NaCl in order to remove the last traces of remaining colipase. Iodination was performed by the triiodide method [8]. Approximately one atom of iodine/procolipase molecule was found to be incorporated. [125 I]colipase was obtained by trypsin activation of [125 I]procolipase [3]. The iodinated cofactors retained 50% of the original colipase activity at pH 6.5 on a tributyrin assay in the presence of TDC.

Surface barostat technique

Assays were performed in a special "zero-order" trough drilled into a Teflon block [9]. The reaction compartment contained 230 ml of solution with a total surface of 123 cm². The subphase of the reaction compartment was thermostatically maintained at 25°C $\pm$ 0.5°C by circulating water in an immersed glass coil. The subphase was continuously agitated with one magnetic stirrer (250 rev/min). 1,2-dilaurin, in CHCl₃ solution ($\approx$ 1 mg/ml), was spread over the entire subphase with a micro-liter syringe. Several minutes were allowed to pass for solvent evaporation. The 1,2-dilaurin film was first equilibrated at the required surface pressure, between 32.5 and 40 dynes/cm. A volume of a 100 mM taurodeoxycholate stock solution was injected into the aqueous subphase of the reaction compartment to give a final concentration of 2.0 mM. Surface pressure rose immediately and then stabilized 3-10 min after the injection of bile salts. Surface pressure was then regulated with the barostat. All injections were performed behind a small Teflon bar fixed in the reaction compartment in order to avoid crossing the lipid film with the tip of the pipet. The colipase sample was always injected 1 min after lipase.

Kinetics were recorded for 15-60 min. The film was then collected and an aliquot of the bulk phase was sampled as described earlier [10].

Before each experiment, the trough was cleaned with ethanol, rinsed several times with tap water and finally with distilled water. The aqueous subphase was systematically composed of 10 mM Tris HCl buffer, pH 8.0, 150 mM NaCl and 1 mM CaCl_2 . Distilled water was prepared from alkaline KMnO_4 in an all-glass apparatus. Residual surfac-active impurities were removed before each assay by sweeping and suction of the surface.

RESULTS

To study the action of lipase on rac-1,2-dilaurin monomolecular films in the presence of 2 mM TDC it is necessary to work at surface pressures above 30 dynes/cm due to the high equilibrium surface pressure of the bile salts. Because of its high collapse pressure, rac-1,2-dilaurin is thus a suitable substrate for lipase in the presence of bile salts [11].

As is seen from the surface pressure recording, dashed curve in Figure 1, there is an increase in surface pressure when TDC is injected into the subphase due to the penetration of TDC molecules into the film. After the rise in pressure there follows a slow dissolution of the film which is compensated by the movement of the barrier shown in the two lower continuous tracings (Figure 1). In this mixed film system at high surface pressure, lipase, with or without procolipase, did not show any hydrolytic activity (Figure 2b). After injection of lipase and colipase the hydrolysis of the dilaurin-TDC mixed film proceeds with kinetics characterized by the lag time τ . Figure 2a and b show, respectively the pressure-lag time and pressure-activity profiles of lipase hydrolysis of mixed dilaurin-TDC films. In the pressure range 32-25 dynes/cm, procolipase is able to activate lipase. However, this activating effect is progressively decreased to zero between 35

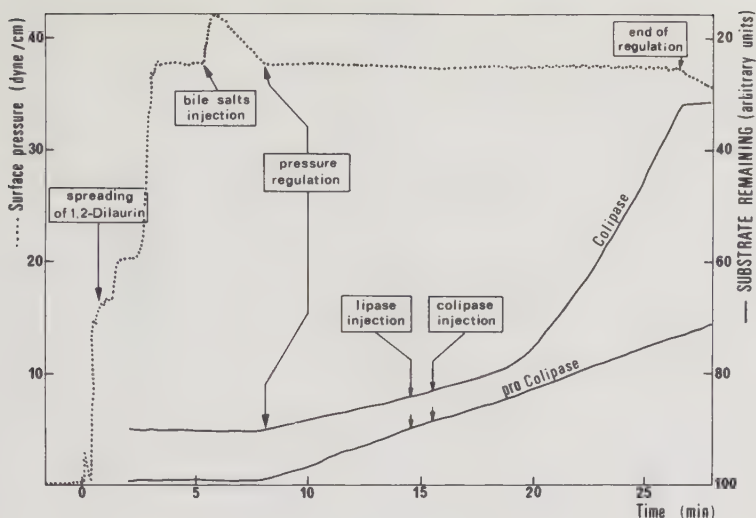


Figure 1. Kinetics of the hydrolysis by pancreatic lipase (0.9 nM) of a rac-1,2-dilaurin film under isobaric conditions (37.5 dynes/cm) and in the presence of taurodeoxycholate (2.0 mM). Experiments performed with colipase (8 nM, middle curve) and procolipase (8 nM, lower curve). The dotted line (upper curve) represents the surface pressure recording. All curves are original kinetic tracings from the barostat recorder. One arbitrary unit of substrate remaining corresponds to 1.55 nmoles of rac-1,2-dilaurin.

and 40 dynes/cm. A concomitant increase in lag time from 7 min at 32 dynes/cm to 17 min at 35 dynes/cm was observed. At 37.5 dynes/cm the lipase activity was low and a steady state was not reached after 60 min. Colipase, on the other hand has different properties. Upon increasing the surface pressure, lipase activity increased, while the lag time stayed almost constant and low, in the pressure range studied.

Under no conditions could lipase activity be detected in the presence of a 2 mM solution and in the absence of (pro)colipase (Figure 2b).

Binding studies of [125 I]procolipase and [125 I]colipase in the presence or absence of lipase are shown in Figures 2c and 2d, respectively. One can note a difference in the binding properties between pro- and colipase in the absence of lipase.

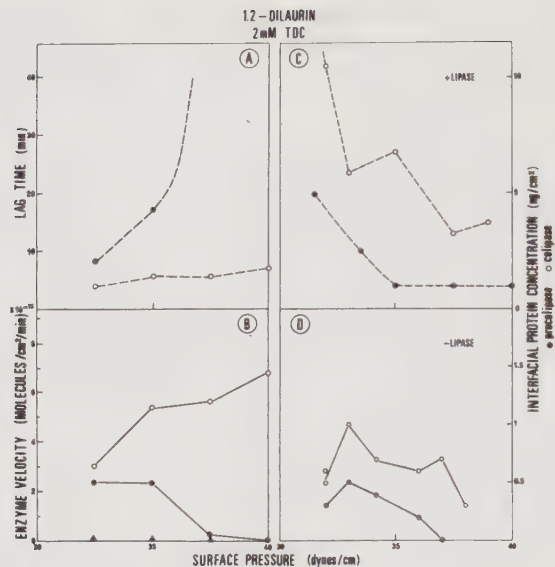


Figure 2. Lipase activity on rac-1,2-dilaurin films (panel B) and lag time, τ (panel A) as a function of surface pressure. Experiments were performed in the presence of 2.0 mM taurodeoxycholate in the bulk phase, and in the presence of pancreatic lipase B (0.9 nM) (▲) and procolipase (8 nM) (●) or colipase (8 nM) (○). Binding of [¹²⁵I]procolipase (21 nM) (●) and [¹²⁵I]colipase (21 nM) (○) as a function of surface pressure in the absence of pancreatic lipase (panel D) and after 10-20 min of hydrolysis by pancreatic lipase B (16 nM) (panel C)

This difference, and the amount bound (pro)colipase, is larger in the presence of lipase and after hydrolysis has been going on, *i.e.* lipolytic products has been formed. The activation of lipase by radiolabelled procolipase and colipase is less efficient than that of the corresponding unlabelled proteins (data not shown). However, its dependence of the surface pressure is similar to what was observed using unlabelled cofactors (Figure 2b).

DISCUSSION

The activation of procolipase by trypsin should be seen in relation to the general function of the enzymes of the pancreatic juice, *i.e.* a fast and efficient degradation of the chyme to molecules absorbable by the enterocytes. The trypsin cleavage of the N-terminal part of the pancreatic zymogens generally yields active molecules better fitted for their metabolic role but at the same time harmful for the parenchymal tissues. Accordingly the activation of procolipase by trypsin results in the formation of a molecule with better potential lipase activating properties. The present investigation using rac-1,2-dilaurin-TDC mixed monomolecular films as lipase substrate is another evidence that such a difference between procolipase and colipase exists.

At 32.5 dynes/cm the lipase activity in the presence of colipase is only 25% higher than in the presence of procolipase. This situation is comparable to that prevailing in bulk system using tributyrin as substrate in the presence of TDC [3]. At higher surface pressures the procolipase activation of lipase decreases with a concomitant increase in lag time, attributable to a slow protein penetration into the film (Figure 2a). This decrease in activity could be due to a decrease in binding of procolipase to the film (Figure 2c). At 40 dynes/cm the lipase activity is zero and could not be restored even with a tenfold increase in procolipase concentration. In contrast, the colipase activation of lipase increases with surface pressure (Figure 2b) although there is a decrease in the amount of colipase bound to the film (Figure 2c). This might indicate that the activation of colipase also affects the binding of lipase to colipase at the interface and/or the turnover number of enzyme [12,13]. This phenomenon will be further investigated using simultaneously radiolabelled lipase and colipase. Nevertheless the better binding properties of colipase compared to procolipase are illustrated by the large difference in lag time between the two proteins (Figure 2a). The increase in binding of the both colipases after hydrolysis started clearly

indicates that reaction products at the interface enhance the binding of colipase.

Apparently at a surface pressure of 40 dynes/cm, procolipase does not activate lipase against a rac-1,2-dilaurin substrate, while colipase does. Is this relevant to the *in vivo* situation? The diglycerides certainly are one of the principal physiological substrates of pancreatic lipase. It is well documented that the dietary triglycerides are partially degraded to diglycerides and fatty acids already when they enter the duodenum due to the action of the lingual lipase [14-18]. These glycerides are emulsified by the fatty acids, alimentary phospholipids and mixed with bile. All these compounds are likely to form highly packed structures. The colipase molecule thus has to overcome these barriers by having efficient binding properties.

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PRODUCT ACTIVATION OF PANCREATIC LIPASE
LIPOLYTIC ENZYMES AS PROBES FOR LIPID/WATER INTERFACES

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SUMMARY

During the action of pancreatic lipase and colipase on rac-1,2-dilaurin monolayers in the absence of bile salts, biphasic kinetics were observed under conditions of high lipid packing. Similar kinetics have earlier been reported using phospholipid emulsified triolein droplets (B. Borgström, Gastroenterology 78 (1980) 954-962). These kinetics are characterized by a lag time τ_d , dependent on products accumulation at the substrate/water interface. This lag time is differentiated from the previously described enzyme concentration independent lag time τ_i , in systems of low lipid packing (Verger, R., Mieras, M.C.E. and de Haas, G.H. (1973) J. Biol. Chem. 248, 4023-4034). Both τ_i and τ_d reflect a rate limiting step due to the slow enzyme penetration into the substrate interface.

The variation of τ_d under different conditions (change in pH and concentration of Ca^{2+} , enzyme, BSA and lipolytic products) lead us to propose a model for the product activation during lipolysis. We will discuss the use of the pancreatic lipase-colipase system to probe the lipid packing of emulsified triglyceride particles and lipoproteins using τ_d as a reference value.

Abbreviations used:

PC: phosphatidylcholine; di- C_{12} -PG: didodecanoylphosphatidylglycerol; di- C_{12} -G and rac-1,2-dilaurin: rac-1,2-didodecanoylglycerol; BSA: bovine serum albumin; PLA_2 : phospholipase A_2 (E.C. 3.1.1.4).

INTRODUCTION

Pancreatic lipase (E.C. 3.1.1.3) catalyzes the hydrolysis of triacylglycerols to fatty acids and 2-monoglycerides (1). In the presence of bile salts, lipase has an absolute requirement for a small protein cofactor, colipase, to exert its catalytic function (for rev. see 2,3). It has been suggested that one role of colipase is to shift upwards the surface pressure range where lipase can penetrate its substrate (4).

A kinetic model for the penetration and reaction steps of lipolytic enzymes acting at lipid/water interfaces has been proposed (5). The kinetics of lipolysis are simply described by a reversible, rate limiting, penetration step of the enzyme into the interface characterized by a lag time τ_i , before the steady state reaction is reached.

The *in vitro* hydrolysis of phospholipid emulsified triolein particles, Intralipid, by pancreatic lipase, in the presence of colipase, has recently been shown to follow an unusual biphasic kinetics. An initially slow accelerating phase preceded a sudden phase of high hydrolytic rate. The duration of the low activity phase was shown to be dependent, among other factors, on the concentration of enzyme, fatty acids and calcium ions in the system (6).

In order to get new insights into these unusual kinetics, we have adopted the monolayer technique which is a powerful tool in such type of investigations (7). We will report here kinetic observations of the action of the lipase/colipase system on highly packed lipid surfaces. We will discuss the results in view of earlier proposed kinetic models and also the possibility to probe natural lipid structures by the use of the lipase/colipase system.

MATERIALS AND METHODS

Lipids

Rac-1,2-didodecanoyl glycerol was purchased from Serdary Research Laboratories (Ontario, Canada) and showed one major spot by thin layer chromatography. Egg-PC was purified from egg as described (8). Di-C₁₂-PG was a gift from Dr. A. Slotboom (Utrecht, Holland). Monoolein was synthesized in the laboratory (9). Oleic acid was from British Drug House and purified on an ion exchanger. Intralipid was a product from Vitrum (Stockholm, Sweden). It is available as an emulsion of 200 g fractionated soybean triacylglycerol, 12 g egg-PC and 25 g glycerol/1000 ml. All other chemicals were of analytical grade.

Proteins

Porcine pancreatic colipase and procolipase were prepared as described previously (10,11). Porcine pancreatic lipase B was purified according to published methods (12), with an additional Sephadex G-100 filtration at pH 9.2 in order to remove remaining colipase contaminations (unpublished). Procolipase was radiolabeled with ¹²⁵I using the triiodide method described earlier in the case of pancreatic PLA₂ (13). Approximately one atom of iodine procolipase molecule was found to be incorporated. [¹²⁵I]Colipase was obtained by trypsin activation of [¹²⁵I]procolipase (11). The iodinated cofactors retained 50% of the original colipase activity at pH 6.5 in a tributyrin assay in the presence of TDC.

Surface barostat techniques

Assays were performed in a special "zero-order" trough (14) drilled into a Teflon block. The reaction compartment contained 230 ml of solution with a total surface of 123 cm². The subphase of the reaction compartment was thermostatically maintained at 25°C ± 0.5°C by circulating water in an immersed glass coil. The subphase was continuously agitated with one magnetic stirrer (250 rev/min). 1,2-dilaurin in CHCl₃ solution

(≈ 1 mg/ml) was spread over the entire subphase with a micro-liter syringe. Several minutes were allowed to pass for solvent evaporation. All injections into the subphase were performed behind a small Teflon bar fixed in the reaction compartment in order to avoid crossing the lipid film with the tip of the pipet.

Kinetic were recorded for 15-60 min. When required the film was then collected and an aliquot of the bulk phase was sampled as described (15).

Aqueous subphase

Before each experiment, the trough was cleaned with ethanol, rinsed several times with tap water and finally with distilled water. The aqueous subphase was composed of 10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl and 1 mM CaCl_2 . Distilled water was prepared from alkaline KMnO_4 in all-glass apparatus. Residual surface-active impurities were removed before each assay by sweeping and suction of the surface.

pH-stat technique

Lipolysis of the Intralipid emulsion was determined titrimetrically using a Mettler automatic titration system (Mettler Instruments AG, Zürich, Switzerland) as described (16). The incubation solution was composed of 0.5 ml Intralipid^R emulsion in 10 ml of a 2 mM Tris-maleate buffer pH 8.0 and 150 mM in NaCl and 1 mM CaCl_2 .

RESULTS

Figure 1 shows a plot of the enzyme activity vs. surface pressure of pancreatic lipase acting on rac-1,2-dilaurin monomolecular film. As is seen, a sharp decrease in activity is observed in the pressure range 30-33 dynes/cm. Such decrease in activity in a narrow surface pressure range is typical for enzymes acting at water/lipid interfaces (17). This surface pressure range is slightly shifted to higher values in the

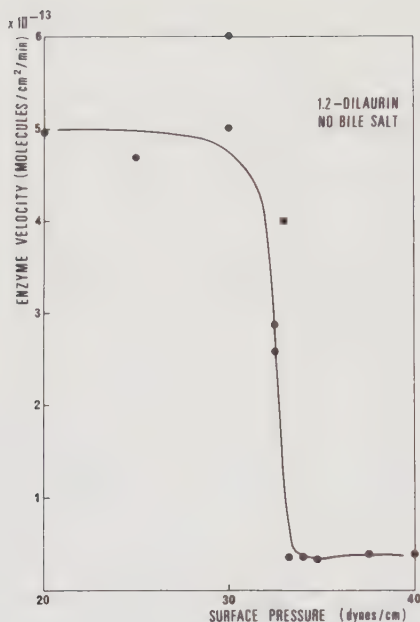


Figure 1. The lipase activity as a function of surface pressure on rac-1,2-dilaurin monomolecular film. Experimental conditions: pancreatic lipase (24 pM), 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM CaCl_2 , 25°C.

presence of colipase (13). A typical recording of the lipase hydrolysis of rac-1,2-dilaurin at a surface pressure of 28 dynes/cm is shown in Figure 2a. This kinetic is characterized by a progressive increase in hydrolytic rate according to a first order process until a steady state reaction is reached. From the kinetic recording a lag time τ can be determined by extrapolation. This lag time reflecting the rate limiting step in the adsorption of the enzyme has been shown to be independent of enzyme concentration (5) and will be labelled, τ_1 , lag time independent of enzyme concentration. Plotting the value $(P'-P)$, as defined in Figure 2a, vs. time in a semilogarithmic way, gives a straight line, Figure 2a (insert) indicative of the first order kinetics of the process. This semilogarithmic plot of the kinetics has been used as a criteria to define τ_1 in our experiments, both in the monolayer and in the emulsion systems.

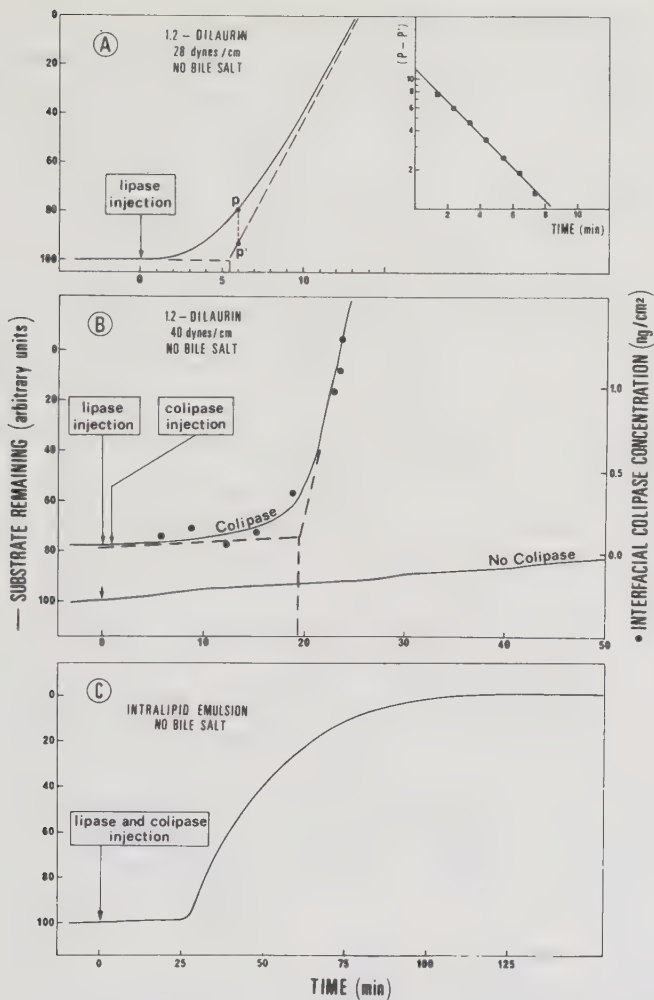


Figure 2. A. Kinetic tracing of the hydrolysis of rac-1,2-dilaurin monomolecular film by pancreatic lipase (24 pM), under isobaric conditions, 28 dynes/cm. The continuous curve is the tracing of the barostat recorder. The broken lines are computed according to Verger *et al.* (5). The intercept between the asymptote and the broken line axis is the lag time τ_l . Insert: The semilogarithmic plot of $(P-P')$ vs. time. Experimental conditions as in Figure 1.

B. Kinetic tracings of the hydrolysis of rac-1,2-dilaurin monomolecular film by pancreatic lipase (0.9 nM) in the absence (lower graph) and presence of [125 I]colipase (8 nM) under isobaric conditions, 40 dynes/cm. The intercept between the broken lines gives the lag time τ_l . The dots (•) indicate the amount of [125 I]colipase bound to the film. Experimental conditions as in Figure 1.

C. Kinetic tracing of the hydrolysis of a phospholipid stabilized triolein emulsion by pancreatic lipase (0.2 μ M) in the presence of colipase (0.5 μ M). Experimental conditions: 100 mg triolein emulsion in 10 ml 2 mM Tris-maleate buffer pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 37°C.

At a surface pressure of 40 dynes/cm the lipase activity is very low (Figure 1 and 2b). In the presence of colipase, however, the reaction process is different (Figure 2b), from what was observed previously at 28 dynes/cm (Figure 2a). After a certain time of low hydrolytic rate a short accelerating phase leads to a high reaction rate. This sudden increase in hydrolytic rate is accompanied by a concomitant increase in colipase binding to the interface (Figure 2b). By extrapolating the pseudo steady state to the time axis, a lag time can be derived, which varies with pH and decreases with the addition of Ca^{2+} ions into the subphase (Figure 3a,b). Furthermore, this lag time decreases with increasing concentration of lipase, colipase and lipolytic products such as oleic acid and monoolein. As this lag time is dependent upon the factors influencing the presence of products in the interface, we will name it τ_d , lag time, product dependent. τ_d has earlier been observed during the hydrolysis of phospholipid stabilized triolein emulsions by pancreatic lipase. For the purpose of comparison the kinetics of such an experiment is shown in Figure 2c. A comparative study of the effects of different additives on the τ_d in the rac-1,2-dilaurin monolayer system and the triolein emulsion system is summarized in Figure 5.

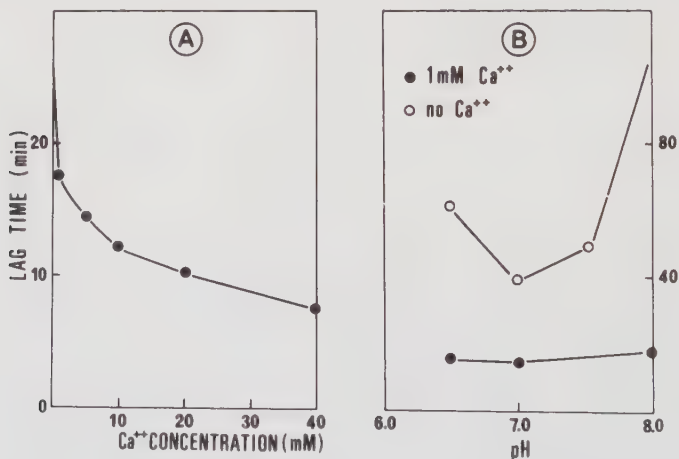


Fig. 3. A. The lag time, τ_d , as a function of calcium concentration. In the absence of calcium no activation could be observed during the time course of the experiments. Conditions: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 25°C. B. The lag time, τ_d , as a function of pH in the presence (●) and absence (○) of 1 mM CaCl_2 . Conditions as above.

Biphasic kinetics are also observed during the hydrolysis of chylomicrons by pancreatic lipase. Comparable τ_d values were obtained using chylomicrons and triolein emulsion containing equal triglyceride amounts, Table I.

TABLE I

Lag time, τ_d , obtained during the hydrolysis of chylomicrons and a triglyceride emulsion (Intralipid) by pancreatic lipase in the presence of colipase.

	Lag time, τ_d (min)
Emulsion (Intralipid)	
30 μ moles Triolein	47
Chylomicrons ^{a)}	
30 μ moles triolein	37

Incubation assay: 10 ml 2 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM CaCl_2 . Temp. 40°C, 0.1 μ M colipase.

a) Rat thoracic duct chyle was collected on ice and dialyzed against an incubation buffer over night.

In order to explain the difference in τ_d between pro-colipase and colipase, binding studies were performed. The binding of [125 I]procolipase and [125 I]colipase to monolayers of egg-PC, di- C_{12} -PG and rac-1,2-dilaurin (di- C_{12} -G) is shown in Figure 4a,b and c. As is seen a difference in the binding of the two cofactors is observed on charged di- C_{12} -PG and zwitterionic egg-PC monolayer while on the electrically neutral rac-1,2-dilaurin monolayer film no difference was observed. Using three times less cofactor in the assay three times less was observed to bind to the monolayer film (data not shown). Both

procolipase and colipase are weakly tensioactive increasing the surface pressure less than 1 dyne/cm in the surface pressure range studied. Procolipase and colipase did not differ in this respect.

DISCUSSION

Pancreatic lipase as a probe for lipid interfaces

The monolayer technique and the bulk titrimetrical techniques have been used extensively in the kinetic studies of lipolytic enzymes. Although complementary in many aspects the two methods have some unique features. The fat emulsion is the natural form in which the substrate is found *in vivo*. This emulsion interface is composed apart from the glycerides of amphiphilic molecules partitioned between the water and lipid phase according to their tensioactive properties. It is, however, impossible to determine the lipid composition, lipid packing or surface pressure of such a lipid/water interface. Such parameters can be obtained with the monolayer technique.

In the present investigation we have observed lag times τ_d , with the monolayer technique using rac-1,2-dilauryl at high surface pressure, originally found in the emulsion system of triolein-phospholipid particles. We have confirmed and extended the studies made with these emulsions and compared the effects of different additives on τ_d . As is seen in Figure 5 there is an excellent agreement between the two methods. From this correspondence it is reasonable to assume that the molecular basis responsible for the existence of τ_d is the same in the two systems.

As τ_d is not observed at low surface pressure (Figure 2a), or in emulsion systems such as dispersed tributyrin in water (11), high lipid packing corresponding to high surface pressure seems to be a necessary condition to observe these types of lag times. The high surface pressure in a phospholipid stabilized triolein emulsion is determined by the packing density of the phospholipid monolayer surrounding the triglyceride

droplets. In the monolayer system, the surface pressure is regulated at will by the moving teflon barrier.

Attempts have been made to determine the surface pressure in biomembranes by comparing the hemolysis of red blood cells by the enzymatic activity of different phospholipases against phospholipid monolayers at different surface pressure (19). By analogy, τ_d in the rac-1,2-dilaurin system can be used to probe the surface pressure in emulsion systems using lipase/colipase as analytical tools, as exemplified here using chylomicrons as lipase substrate, Table I. The surface pressure value of 40 dynes/cm in the rac-1,2-dilaurin system cannot be strictly extrapolated to the emulsion or chylomicron interface, but a value in that order seems reasonable. As an extension of this study it is possible to use the lipase/colipase monolayer system to further investigate the surface properties of natural lipoproteins using τ_d as a reference.

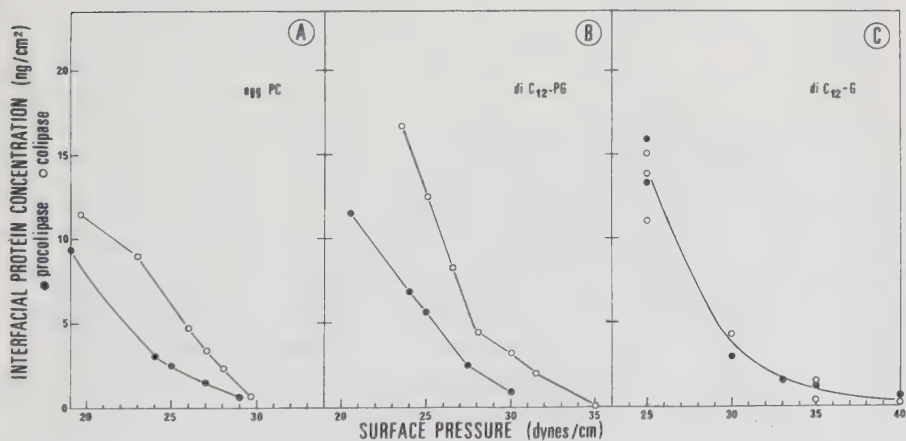


Fig. 4. Binding of [¹²⁵I]procolipase (●) and [¹²⁵I]colipase (○) to a monomolecular film of A. egg-PC B. di-C₁₂-PG and C. rac-1,2-dilaurin (di-C₁₂-G). The cofactors were injected into the subphase, final concentration 4.5 nM. After 10 min the surface was swept with a Teflon bar and radioactivity measured as described (15).

Product activation during lipolysis

The well characterized fatty acid inhibition of lipolysis has been explained as an effect of product accumulation at the interface, making the substrate inaccessible to the enzyme (20). This effect was, however, observed when a substantial amount of substrate had been hydrolyzed. In the situation where such an inhibition was observed usually product acceptors (Ca^{2+} , BSA, bile salts) were not present. In the present situation of product activation only a small amount of the highly packed substrate has been hydrolyzed before the high hydrolysis rate was observed. As biphasic kinetics were not observed at a low surface pressure, a high lipid packing seems to be essential to observe these effects. We interpret this product activation as a consequence of an increased binding of colipase/lipase to the interface (Figure 2b). This binding can be described by the use of the partition coefficient of colipase k_p/k_d^* between the bulk and lipid phases. k_p/k_d is constant for a fixed composition of the interface and is characterized by a lag time τ_i , described earlier (5,14). On highly packed lipid/water interfaces, devoid of lipolytic products colipase is first partitioned in favour of the bulk phase. However, as small amounts of products are formed and remain transitorily at the interface, the interfacial composition changes. This change in the interface increases the k_p of colipase, leading to an increase in lipase binding with a subsequent increase in product formation. The kinetics of product activation and thus, τ_d , are characterized by a time dependent change of k_p due to a time dependent change of the interfacial chemical composition. This change in k_p does not have to be linear with product concentration at the interface. A certain interfacial concentration level of products might induce a large change in k_p .

* k_p - penetration rate constant

k_d - desorption rate constant

for details see ref. (5)

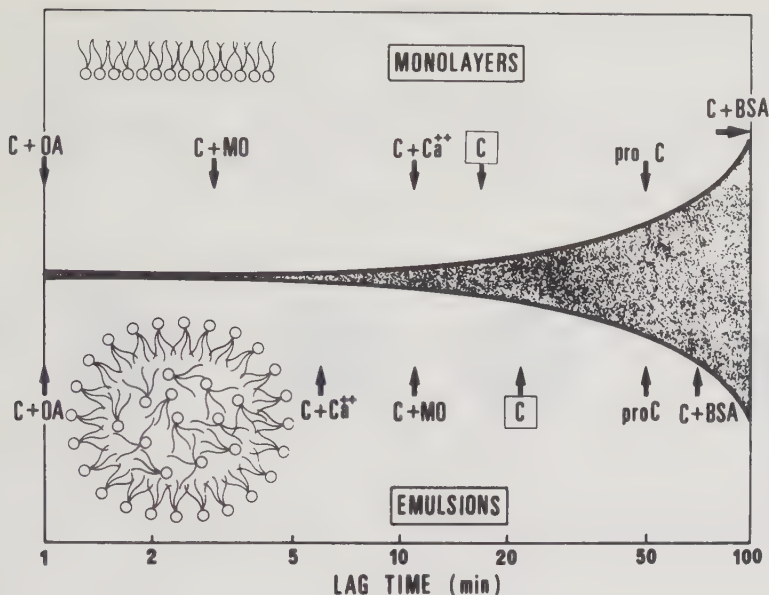


Fig. 5. The product dependent lag time τ_d as a function of different additives during the hydrolysis of a diglyceride monolayer at high surface pressure and a phospholipid stabilized triolein emulsion.

Monolayer conditions: rac-1,2-dilaurin. Isobaric hydrolysis at 40 dynes/cm by lipase (0.9 nM) in the presence of colipase (C) (8 nM) and procolipase (proC) (8 nM).

Emulsion conditions: 100 mg triolein phospholipid emulsion (Intralipid) in 10 ml buffer pH 8.0, 150 mM NaCl, 1 mM CaCl_2 . 0.2 μM lipase and 0.5 μM colipase.

When added: Monoolein (MO) was 4 mol-%, oleic acid (OA) 4 mol-%, Ca^{2+} 10 mM, and BSA 0.1 μM .

The molecular interpretation of τ_d can so far only be tentative. Our picture of the events taking place are summarized in Figure 6. At a highly packed lipid/water interface colipase/lipase are adsorbed following a first order process characterized by a lag time τ_1 . With time products are formed enhancing the lipase/colipase binding which will be reflected by an increase in lipolytic activity. It has been reported that fatty acids might form small clusters of 10-20 molecules in lipid bilayers and monolayers (21). Furthermore, fatty acid clusters are stabilized by the presence of calcium ions and the size of the clusters was found to be 50-60 Å (22). Our data are compatible with the formation of such clusters of fatty acid calcium soaps

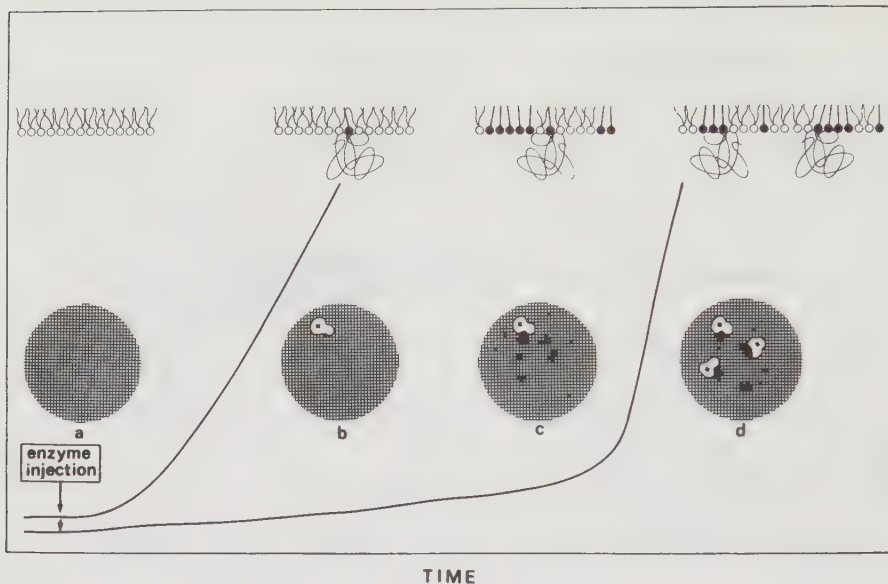


Fig. 6. A tentative picture of the molecular events taking place at the highly packed (high π) lipid/water interface (a) during product activation of lipolysis. The enzyme (cofactor) initially partitions between the bulk water and lipid interface (b). Hydrolysis starts and products transiently stay at the interface forming clusters or microheterogeneities (c). At the boundaries of these clusters the binding of the enzyme is enhanced with a resultant increase in hydrolytic rate (d). At low surface pressure (low π) only conditions a and b prevail. The corresponding kinetic tracings are also drawn (left - low π , right - high π).

at the interface. At the boundaries of these clusters discontinuities might exist, as pictured in Figure 6. The better penetrating capabilities of colipase as compared to procolipase towards charged and zwitterionic interfaces can explain the observed differences in τ_d in the presence of either of the two proteins (Figure 5) (11). Although no difference in binding was observed on pure rac-1,2-dilaurin monolayer film (Figure 4c); the fatty acid and fatty acid-calcium soaps formed during the hydrolysis might induce a change in the charge properties of the surface and thus increase the binding of the cofactors and preferentially colipase.

BSA, which is known to bind fatty acid and monoglycerides, will cause a fast desorption of products from the interface and consequently the disappearance of the clusters, and thus an increase in τ_d (Figure 5).

The τ_d is apparently not only observed during the action of pancreatic lipase-colipase on highly packed lipid structures. Quarles and Dawson demonstrated that phospholipase D acted in a similar manner on phosphatidylcholine monolayers at high surface pressure. The long lag times observed could be decreased by addition of phosphatidic acid and calcium ions (23).

Roholt and Schlamowitz studied the action of phospholipase A_2 from *Crotalus durissus terrificus*. τ_d was observed when dimyristoyl lecithin was used as substrate and could be decreased by the presence of myristoyl lysolecithin (24).

More recently Upreti *et al.* showed that the hydrolysis of phospholipid bilayers by bee venom phospholipase A_2 is enhanced by the presence of different alkanols in the bilayer due to the free space introduced in the bilayers where the enzyme act (25). Upreti and Jain further demonstrated that the hydrolysis of PC-bilayers by bee venom PLA_2 proceeds according to a biphasic kinetic (26).

Tinker and Wei have presented a model to describe the biphasic kinetics of *Crotalus atrox* PLA_2 activity on phospholipids in the gel phase. However, in contrast to our explanation the authors introduced the postulate of product stimulated desorption of the enzyme to account for the dramatic stimulation of the reaction induced by both lyso-PC and fatty acids (27). These effects could, however, as well be explained by our model of product activation developed for the lipase-colipase system. The answer to these contradictory interpretation could be given by direct studies of the *Crotalus atrox* PLA_2 during the lag phases as previously reported by Pattus *et al.* in the case of the pancreatic PLA_2 (28).

The physiological relevance of the product activation of the pancreatic lipase/colipase lipolysis is evident. The dietary glycerides are already hydrolyzed up to 30% by the action of the lingual lipase in the stomach (29-33). The surface of the triglyceride emulsion is thus "activated" by these fatty acids enhancing the binding of colipase and the initiation of duodenal lipolysis.

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CALCIUM BINDING TO PORCINE PANCREATIC PROPHOSPHOLIPASE A₂ STUDIED BY ⁴³Ca NMR

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1. Introduction

Pancreatic phospholipase A₂ (PLA₂) catalyzes the hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl *sn*-phospholipids [1]. The enzyme is secreted by the pancreas in an inactive form. In the duodenum the zymogen is activated by trypsin cleavage [1]. The enzyme is capable of degrading phospholipids in micellar form, while the proenzyme is only able to hydrolyze monomeric substrates [2,3]. It has been shown that Ca²⁺ are essential for the catalytic properties of both the enzyme and the zymogen. Ca²⁺ binding to both forms has been studied by a variety of physicochemical techniques, showing that Ca²⁺ binds in a 1:1 molar ratio with association constants in the range of $1-5 \times 10^3 \text{ M}^{-1}$ [4,5]. From X-ray crystallographic data the Ca²⁺ binding site has been found to be located in a cavity of the protein surface near Asp₄₉ [6].

Here we report the results of ⁴³Ca NMR measurements of Ca²⁺ binding to prophospholipase A₂ (PPLA₂). It is shown that the method is useful in providing information about: (i) association constants; (ii) the exchange rate of Ca²⁺ (i.e., k_{off}); (iii) dynamics in the Ca²⁺ binding site; and (iv) the apparent *pK* value for the group(s) involved in Ca²⁺ binding.

2. Materials and methods

2.1. Materials

Porcine pancreatic phospholipase A₂ was purified from a fortified preparation obtained as a side fraction during insulin production. The chromatographic steps were performed essentially as in [7]. The pro-

phospholipase obtained showed no activity in an egg yolk assay [7]. Protein concentrations were determined spectrophotometrically using the absorption coefficient $E_{1\%}^{1\text{cm}} = 12.3$ at 280 nm [7]. A 0.086 M CaCl₂ solution was prepared by dissolving CaCO₃ (60% isotopically enriched in ⁴³Ca, Oak Ridge Nat. Lab.) in 0.1 M HCl and the solution was neutralized to a final pH at 7.0.

2.2. Methods and data treatment

The ⁴³Ca NMR spectra were obtained at 17.16 MHz, using a homemade Fourier transform spectrometer [8]. The relaxation of ⁴³Ca can safely be assumed to be exclusively quadrupolar. For correlation times sufficiently short, such that $\omega\tau_c \lesssim 1$, the relaxation can be approximated by a single exponential, corresponding to a Lorentzian line in the Fourier transformed spectrum. When the observed signal is due to metal ions exchanging between two sites, the line shape is no longer Lorentzian, except for very slow and very fast exchange rates. When the intermediate exchange condition applies the full width at half height of the NMR signal, $\Delta\nu$, is not only influenced by the transverse relaxation rates in the two sites and their populations, but also the exchange rate (i.e., k_{off}), cf. the Swift-Connick equation ($x = 1$ indicates longitudinal and $x = 2$ transverse relaxation rates):

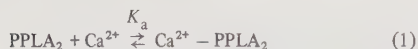
$$R_{x,\text{obs}} = (1 - p_b)R_{x,f} + p_b/(R_{x,b}^{-1} + k_{\text{off}}^{-1})$$

which is valid for p_b values $\ll 1$ [9]. When the Swift-Connick approximation is not fulfilled, a total band-shape analysis is needed to obtain values of k_{off} and $R_{2,b}$. The details of the band-shape analysis will be reported elsewhere. To visualize the agreement

between calculated and observed signals, the full width at half height ($\Delta\nu = R_{2,\text{obs}}/\pi$) will be used in the same way as when the Swift-Connick equation is valid.

3. Results

Fig.1 shows the line-width of the observed ^{43}Ca NMR signal as a function of the $[\text{Ca}^{2+}]$ at a constant [protein]. The broadening of the ^{43}Ca signal is most likely due to Ca^{2+} exchanging with the catalytic site of PLLA₂. The data can be fitted to the model of a simple chemical equilibrium:



The details of the fitting procedure will be published elsewhere. The value obtained for the association constant K_a is $2.5 \pm 1 \times 10^3 \text{ M}^{-1}$ at 24°C .

The temperature dependence of the ^{43}Ca NMR line-width is shown in fig.2. By fitting the data we obtained: $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ and the line-width of the bound ion $\Delta\nu_b = 5 \pm 0.6 \times 10^2 \text{ Hz}$ (corresponding to $R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$) at 24°C . Measurements of the longitudinal relaxation rate, $R_{1,\text{obs}}$, for a 1.7 mM PLLA₂ solution containing 10.0 mM Ca^{2+} (pH 7.0) results in a value of $79 \pm 4 \text{ s}^{-1}$.

The pH dependence of the observed ^{43}Ca NMR line-width is shown in fig.3. The data can be fitted to the model of a single protonation step:

$$\Delta\nu = [\Delta\nu_i]/[1 + 10^{(\text{p}K_{a,i} - \text{pH})}] + C \quad (2)$$

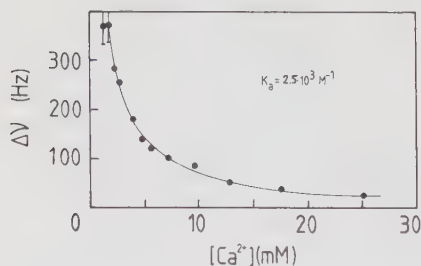


Fig.1. The line-width of the ^{43}Ca NMR signal as a function of the $[\text{Ca}^{2+}]$ for a 2.0 mM PLLA₂ solution (pH 7.5). The spectra were recorded at 24°C . The solid line represents the theoretical curve calculated as described in the text.

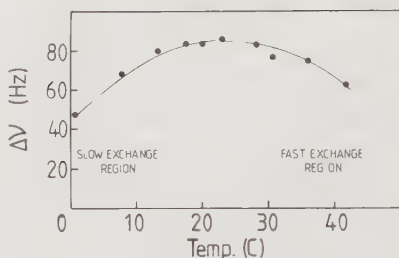


Fig.2. The temperature dependence of the ^{43}Ca NMR line-width for a 1.7 mM PLLA₂ solution (pH 7.4) containing 5.9 mM Ca^{2+} . The solid line is the theoretical curve drawn as described in the text.

In this equation $\Delta\nu_i$ is the step in the line-width associated with the apparent $\text{p}K$ value $\text{p}K_{a,i}$. C is the contribution to the observed line-width that stays unaffected during the titration. In the fitting procedure we obtained the following parameters: $\text{p}K_{a,i} = 5.2$, $\Delta\nu_i = 91 \text{ Hz}$ and $C = 8 \text{ Hz}$.

4. Discussion

Ca^{2+} binding to PLLA₂ has been studied using an equilibrium gel filtration technique [4] and UV difference spectrophotometry [4,5]. In these investigations, association constants in the range $2\text{--}4 \times 10^3 \text{ M}^{-1}$ were reported. The value of the association constant obtained from the ^{43}Ca concentration dependence, $K_a = 2.5 \pm 1 \times 10^3 \text{ M}^{-1}$, is in good agreement with these results.

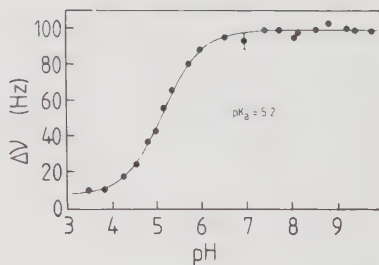


Fig.3. The pH dependence of the ^{43}Ca NMR line-width for a 1.7 mM PLLA₂ solution in the presence of 5.9 mM Ca^{2+} . The spectra were recorded at 23°C . The solid curve represents the model of a single protonation step with an app. $\text{p}K$ of 5.2.

The value obtained for the rate of the dissociation of Ca^{2+} from PPLA_2 (i.e., $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ at 24°C) may be compared with the corresponding figure for rabbit skeletal muscle troponin C (TnC). The off-rate for Ca^{2+} binding to the regulatory sites of TnC ($k_{\text{off}} = 1 \times 10^3 \text{ s}^{-1}$; T. A. et al., unpublished) is of the order of magnitude that one would expect if the on-rate were essentially diffusion controlled. (Taking the association constant of Ca^{2+} to the regulatory sites to be $3 \times 10^5 \text{ M}^{-1}$ [10], we obtain $k_{\text{on}} = 3 \times 10^8 \text{ s}^{-1} \text{ M}^{-1}$.)

In contrast, the on-rate of Ca^{2+} binding to PPLA_2 must be at least two orders of magnitude slower than the on-rates to the regulatory sites of TnC in order to account for the relation between the association constant and the off-rate. This large difference reflects the different nature of the regulatory Ca^{2+} binding sites of TnC on the one hand and that of PPLA_2 on the other. The former sites seem to have a flexible structure, such that the Ca^{2+} binding amino acid residues may easily wrap around an incoming ion. The Ca^{2+} binding site of PPLA_2 seems to be more rigid or generally less accessible to an incoming Ca^{2+} .

The value of the longitudinal relaxation rate, $R_{1,b}$, of the Ca^{2+} at the PPLA_2 binding site may be estimated from the Swift-Connick equation (see above). By using the experimentally obtained values: $R_{1,\text{obs}} = 79 \pm 4 \text{ s}^{-1}$, $R_{1,f} = 0.71 \text{ s}^{-1}$, $p_b = 0.17$ and $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ we may calculate $R_{1,b}$ to be $5.7 \pm 0.8 \times 10^2 \text{ s}^{-1}$.

To obtain an estimate of $R_{2,b}$ we have not followed a procedure similar to that described above for $R_{1,b}$ since chemical exchange effects distort the observed ^{43}Ca NMR signal from a Lorentzian line shape. Instead we have used the value obtained from fitting the variable temperature data: $R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$. From the ratio $R_{2,b}/R_{1,b}$ it is possible to calculate the correlation time τ_c for the bound Ca^{2+} .

Under conditions such that $\omega\tau_c \leq 1$ it may be shown that $[11,12] R_{2,b}/R_{1,b} = (0.3 + 0.5 \tilde{J}_1 + 0.2 \tilde{J}_2) / (0.2 \tilde{J}_1 + 0.8 \tilde{J}_2)$, where $\tilde{J}_1 = (1 + (\omega\tau_c)^2)^{-1}$ and $\tilde{J}_2 = (1 + (2\omega\tau_c)^2)^{-1}$. The quotient $R_{2,b}/R_{1,b} = 2.9 \pm 0.7$ corresponding to $\omega\tau_c = 1.3 \pm 0.3$ or $\tau_c = 12 \pm 3 \text{ ns}$. This value is close to that expected for the rotational diffusion of the entire PPLA_2 molecule. Taking the average hydrodynamic radius to be 2.1 nm this gives $\tau_c = 8 \text{ ns}$ from the Debye-Stokes-Einstein equation [13]. This may be taken to indicate that the Ca^{2+} binding region has a relatively low internal mobility.

From the relaxation rate of the bound $^{43}\text{Ca}^{2+}$

($R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$) and the correlation time ($\tau_c = 12 \text{ ns}$) we may calculate the quadrupolar coupling constant, χ , of the bound $^{43}\text{Ca}^{2+}$ from the expression [11,12] $R_{2,b} = (2\pi^2\chi^2/49)(0.3\tau_c + 0.5\tau_c\tilde{J}_1 + 0.2\tau_c\tilde{J}_2)$ to be $0.8 \pm 0.1 \text{ MHz}$. This value, which is the first to be reported for a Ca^{2+} binding protein may be compared with the quadrupole coupling constants observed for $^{43}\text{Ca}^{2+}$ bound to EDTA (0.5 MHz) and EGTA (2.1 MHz) (T.D., unpublished).

The apparent pK value ($\text{pK}_a = 5.2$) from the pH dependence of the ^{43}Ca line-width probably reflects the ionization of Asp_{49} , the only charged amino acid residue that is directly involved in the Ca^{2+} binding [6]. Our value is in excellent agreement with the value in [14] ($\text{pK}_a = 5.2$) for the active enzyme.

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